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POTENTIAL OF A LACTIC ACID BACTERIUM FOR THE BIOLOGICAL PRESERVATION OF GROUND BEEF

by

© KIMBERLY A. BURNS

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE

OF Master of Science

IN

Food Microbiology

Department of Food Science

EDMONTON, ALBERTA

Fall 1987

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled **POTENTIAL OF A LACTIC ACID BACTERIUM FOR THE BIOLOGICAL PRESERVATION OF GROUND BEEF** submitted by **KIMBERLY A. BURNS** in partial fulfilment of the requirements for the degree of **Master of Science** in **Food Microbiology**.

Michael E. Stiles
.....
Supervisor

.....

.....

.....

Date October 5/87

Dedicated to

MOM, DAD AND KEL

ABSTRACT

The potential for a lactic acid bacterium isolated from vacuum-packaged beef to serve as a biological preserving agent was examined. Lactic isolates were screened for their ability to inhibit the growth of selected indicator organisms on APT agar by direct and deferred methods of antagonism. One bacterium, referred to as isolate #26, was discovered which inhibited the growth of a wide range of organisms. To evaluate the potential of isolate #26 for use as a biological preservative part of this study was devoted to determining the identity of isolate #26, a second part to determining the nature of the inhibitor(s) produced, and a third part to determining if the inhibitor is synthesized and active in ground beef. Isolate #26 is an atypical betabacterium, since it produces only L(+) lactic acid. It was identified as *L. piscicola*, formerly proposed as *L. carnis* (Shaw and Harding, 1985). Inhibition by isolate #26 on APT agar was attributed to the production of a proteinaceous bacteriocin-like substance (Bac #26). Bac #26 was active against species of *Pediococcus*, *Lactobacillus*, *Streptococcus*, *Leuconostoc*, *Bacillus*, *Clostridium* and *Listeria*, but not against the gram-negative organisms tested. On APT agar Bac #26 was produced at temperatures as low as 1°C. However, the antimicrobial effect was not seen on APT agar adjusted to pH 6.0 or less. This suggests that inhibition by Bac #26 production would not occur in meat (pH < 6.0). Inhibition of *S. faecalis* in irradiated meat

occurred only when high inocula (10^9 CFU of isolate #26/g) was used. Therefore inhibition of *Streptococcus faecalis* was likely the result of organic acid production and other factors, possibly physical limitation of growth space. Successful isolation of a strain (isolate # 26) which produces a bacteriocin-like compound demonstrates the potential for isolating an organism suitable as a biological preservative from vacuum-packaged beef.

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1. INTRODUCTION

The lactic acid bacteria (or the lactics) have found an ecological niche in a number of food products. It is commonly known that the lactics are used in the production of fermented foods, such as dairy and meat products, where they contribute to the characteristic aroma, flavor and texture of these products. The lactics also play an important role in the microbial ecology of vacuum-packaged meat (Kitchell and Shaw, 1975; Egan, 1983; Sharpe and Pettipher, 1983).

Aerobically packaged fresh meat has a short shelf-life due to large numbers of gram-negative spoilage bacteria, primarily pseudomonads, which dominate the population at chill temperatures (Erichsen and Molin, 1981; Grau, 1983). Although vacuum-packaging and refrigeration can extend the shelf-life of fresh meat up to eight weeks, or more, once the meat is repacked into gas permeable films, it keeps for only a short period of time (Dainty *et al.*, 1979; Egan, 1983). Methods of preserving this perishable commodity are required.

Various researchers have shown that by inoculation of the lactics, primarily dairy lactics, into fresh aerobically stored meat, the growth of the spoilage flora and also a number of pathogenic microorganisms ("undesirable flora") can be delayed or inhibited (Price and Lee, 1970; Reddy *et al.*, 1970; Reddy and Chen, 1975; Gilliland and Speck, 1975; Newton and Gill, 1978; Raccach *et al.*, 1979; Abdel-Bar and

Harris, 1984). The use of dairy cultures such as *Streptococcus lactis* and *Leuconostoc citrovorum* (Reddy *et al.*, 1970), *Streptococcus diacetilactis* (Gilliland and Speck, 1975), and *Lactobacillus bulgaricus* (Abdel-Bar and Harris, 1984) all required high inocula to achieve an inhibitory effect.

The exact mechanism of the inhibition is not well understood. It is generally agreed, however, that organic acids (Abdel-Bar and Harris, 1984), hydrogen peroxide (Gilliland and Speck, 1975; Adbel-Bar and Harris, 1984) and perhaps antibiotics (Newton and Gill, 1978; Collins-Thompson and Lopez, 1982) produced by the lactics are responsible for inhibition. Inhibition by strong acid production may cause early spoilage of the product through souring, while hydrogen peroxide or hydrogen sulfide production may cause discoloration through greening (Egan, 1983; Sharpe and Pettipher, 1983). Ideally, lactic strains capable of producing broad spectrum antibiotic- or bacteriocin- type compounds at refrigeration temperatures would be effective cultures for retention of fresh meat quality, providing these compounds do no adversely affect the organoleptic properties of the meat. In recent years, bacteriocins have been isolated and purified from the lactics (DeKlerk and Smit, 1967; Upreti and Hinsdill, 1973, 1975; Barefoot and Klaenhammer, 1983; Daeschel and Klaenhammer, 1985).

A suitable lactic strain for use as a biological preservative in meats must not only produce an inhibitory

compound at refrigeration temperatures but also the inhibitory compound must be synthesized and interact effectively with the microorganisms present in the meat. Most of the dairy lactics are mesophilic, that is, they do not grow or grow poorly at refrigeration temperatures, therefore antimicrobial substances which are produced as a by-product of growth will not be formed at refrigeration temperatures. For example *Streptococcus lactis* produces nisin, an antibiotic which is active against a number of gram-positive bacteria (Hurst, 1973). However, if *S. lactis* was inoculated onto refrigerated carcass meats, these mesophilic bacteria would not be able to grow and produce nisin. If the temperature was raised to allow growth of *S. lactis*, nisin would be ineffective against the gram-negative microflora predominating at the higher temperature.

The lactics, particularly the lactobacilli, become the major group of microorganisms on refrigerated vacuum-packaged meat (Roth and Clark, 1972; Reuter, 1975; Sutherland *et al.*, 1975; Savell *et al.*, 1981; Hitchener *et al.*, 1982; Shaw and Harding, 1984). Vacuum-packaged meat is therefore, a rich source of lactics which are capable of growing at refrigeration temperatures. The fact that certain lactics proliferate on vacuum-packaged meat, to the virtual exclusion of all other bacterial types, suggests that among them there may exist suitable candidates for use as biological preservatives.

The objective of this study was to isolate lactic strains from vacuum-packaged fresh meat stored at chill temperatures, capable of producing antimicrobial compounds active against a broad spectrum of indicator organisms, and, subsequently, to evaluate the potential of using such a strain(s) as a tool for retention of fresh meat quality.

2. REVIEW OF LITERATURE

2.1 The Lactic Acid Bacteria

The term lactic acid bacteria (lactics) refers to a diverse group of microorganisms that are capable of producing lactic acid from a fermentable carbohydrate source. A fairly precise definition of this group was given in the classical monograph by Orla-Jensen (1919): "The true lactic acid bacteria form a great natural group of immotile, sporeless, Gram positive cocci and rods which in fermenting sugar chiefly form lactic acid." The lactic acid bacteria were differentiated taxonomically by Orla-Jensen, based on physiological and morphological properties, into the following six genera: *Betabacterium*, *Streptobacterium*, *Thermobacterium*, *Betacoccus*, *Streptococcus* and *Tetracoccus*. Although the physiological and morphological descriptions of the groups, as envisioned by Orla-Jensen, have not been altered significantly, only the genus *Streptococcus* has retained its taxonomic rank. The first three genera, *Betabacterium*, *Streptobacterium*, and *Thermobacterium* now represent subgroups of the genus *Lactobacillus* (Reuter, 1985). The following genera are currently regarded as lactic acid bacteria: *Streptococcus* (group N), *Lactobacillus*, *Pediococcus*, and *Leuconostoc*. These genera can be separated by the characteristics given in Table 2.1. Depending on the prevailing philosophical attitude, the genera comprising the lactics can be merged into a single biochemically similar

Table 2.1. The major characteristics differentiating *Lactobacillus*, *Leuconostoc*, *Pediococcus* and *Streptococcus*.

Genus	Morphology[1]	Gas Production from Glucose[1]	Isomer of Lactate[2]
Streptococcus	cocci pairs/chains	-	L(+)
Pediococcus	cocci pairs/tetrads	-	DL
Leuconostoc	elongated cocci	+	D(-)
Heterofermentative *Lactobacillus*	rods	+	DL
Homofermentative *Lactobacillus*	rods	+	D,L,DL

[1] Data from Sharpe *et al.* (1966)

[2] Data from Stramer (1979)

group, or they can be divided based on morphological differences into two distinct families, Lactobacteriaceae and Streptococcaceae, as is currently in *Bergey's Manual of Systematic Bacteriology* (Kandler and Weiss, 1986).

2.2 Microflora of Vacuum-Packaged Beef

When fresh beef is vacuum-packaged in plastic films of low gas permeability, the atmosphere becomes depleted in O_2 (often less than 1% v/v) and enriched in CO_2 (generally greater than 20% v/v) (Dainty *et al.*, 1979). Vacuum-packaging creates a favorable environment for growth of the lactics as they do not require oxygen for growth and their growth is stimulated by increased concentrations of carbon dioxide (Sharpe, 1981).

The composition of the microflora which develops on vacuum-packaged meat is controlled by a combination of the pH and the gas permeability of the packaging film (Campbell *et al.*, 1979; Egan, 1983; Grau, 1981, 1983). Initially on fresh beef, there is often less than 10 lactics/cm^2 (Egan, 1983). If the pH of the meat is normal (pH 5.4-5.9) and the permeability of the packaging film is sufficiently low (O_2 permeability is less than 100 mL/m^2-24h-atm. at 25°C) the lactics will grow to the virtual exclusion of all other bacterial types (Roth and Clark, 1972; Dainty *et al.*, 1979; Erichsen and Molin, 1981; Egan, 1983). While the lactics dominate the microflora of vacuum-packaged fresh meat stored at chill temperatures, Enterobacteriaceae and facultative

anaerobes such as *Brochothrix thermosphacta* often form a significant portion of the total population (Dainty *et al.*, 1979; Grau, 1983). In Figure 2.1 the development of a "typical" microflora on beef of normal pH, vacuum-packaged in a film of low oxygen permeability is illustrated. Of the gram-negative bacteria present during the early stages of storage, the pseudomonads are the most common group (Dainty *et al.*, 1979). The Enterobacteriaceae, mainly represented by *Serratia liquefaciens*, are rarely detected during the first one or two weeks of storage, but they become the most common gram-negative bacterium after four weeks of storage (Dainty *et al.*, 1979; Egan, 1983). After four weeks of storage the Enterobacteriaceae may constitute 10% of the meat microflora (Dainty *et al.*, 1979). Reports on the growth of *B. thermosphacta* on vacuum-packaged beef have been inconsistent. Pierson *et al.* (1970) and Roth and Clark (1972) did not detect *B. thermosphacta*, while others (Sutherland *et al.*, 1975; Dainty *et al.*, 1979; Erichsen and Molin, 1981) reported growth to ca. $10^6/cm^2$. Campbell *et al.* (1979) and Grau (1980, 1981) showed that *B. thermosphacta* will not grow anaerobically on meat at pH 5.8 or lower and suggested that the varied reports on its growth are due to differences in the pH of the meat and/or the permeability of the packaging materials used. There is increased growth of the Enterobacteriaceae and of *B. thermosphacta* on meat at pH $\geq$ 5.9 and/or packaged in a film of high oxygen permeability (Dainty *et al.*, 1979).

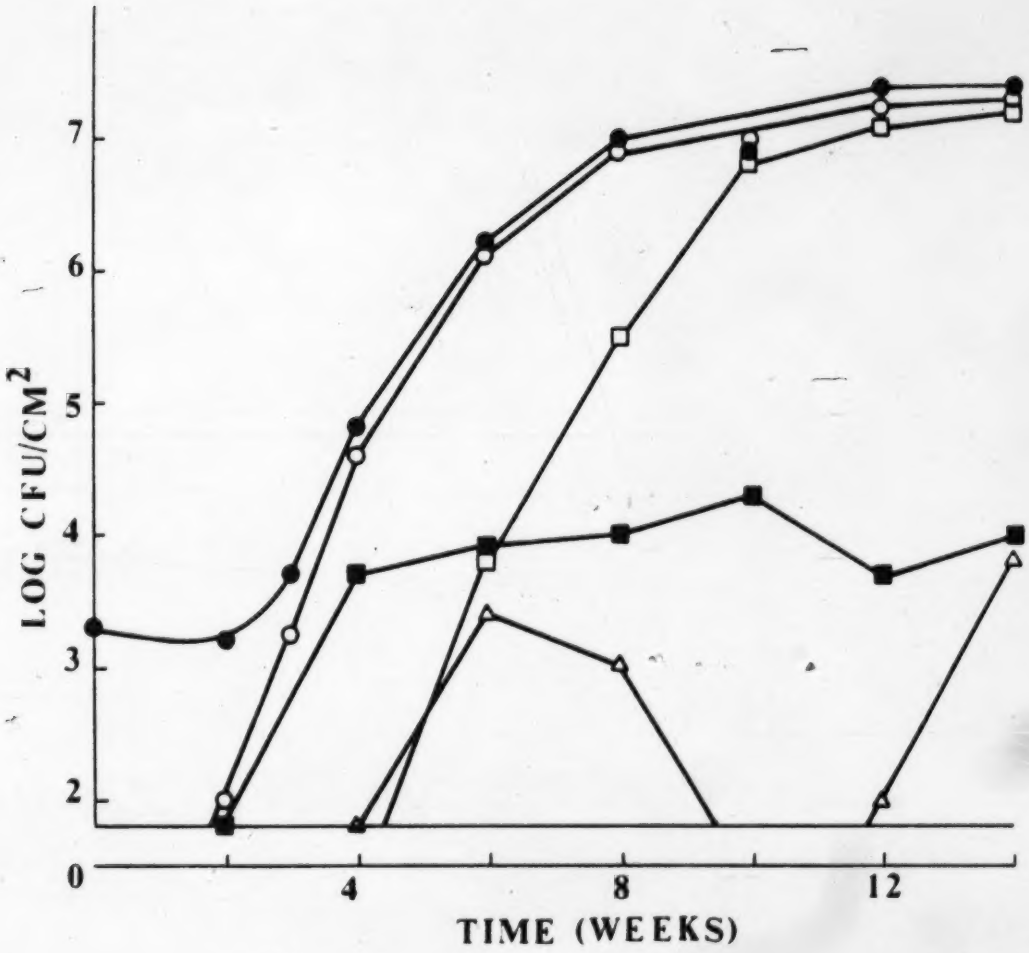


Figure 2.1. Development of microflora on beef of normal pH (5.4-5.9) vacuum packaged in a film of low oxygen permeability during storage at 1°C (Adapted from Egan, 1983).
KEY: (●)total viable count; (○)lactics count on MRS agar, and (□)on Rogosa SL agar, (△)*B. thermosphacta*; (■)gram-negative bacteria.

Not only does the composition of the microflora vary with the pH of the meat and the permeability of the packaging film, but also within the pack between the lean and adipose tissue. Grau (1983) found that *B. thermosphacta* and the Enterobacteriaceae grew more rapidly on the fat surfaces of vacuum-packaged beef during the initial stages of storage. Although *B. thermosphacta* cannot grow anaerobically on lean tissue at pH less than 5.8, growth continues on the adipose tissue when oxygen becomes insufficient for aerobic growth in the vacuum-package (Grau, 1983). This lead Grau (1983) to suggest that within packs of vacuum-packaged meat there are likely several ecologically different environments.

2.2.1 Identity of the lactic acid bacteria isolated from vacuum-packaged beef

Most studies on the identity of the lactic acid bacteria from vacuum-packaged beef found that lactobacilli are the major group (Reuter, 1975; Dainty *et al.*, 1979; Hitchener *et al.*, 1982; Holzapfel and Gerber, 1983; Shaw and Harding, 1984). Dominance of the microflora by *Leuconostoc* spp. has been reported in a few studies (Patterson and Baird, 1977; Savell *et al.*, 1981). In spite of the many described species of lactobacilli, it is generally difficult to identify strains of *Lactobacillus* isolated from meat, presumably because the documented descriptions and schemes of identification are based on isolates from other sources

(Kitchell and Shaw, 1975). Most lactobacilli isolated from vacuum-packaged beef have not been identified or could not be identified at the species level. Such terms as "unclassifiable" or "atypical" streptobacteria or betabacteria have been used to refer to these isolates (Kitchell and Shaw, 1975; Reuter, 1975; Hitchener *et al.*, 1982; Holzapfel and Gerber, 1983; Shaw and Harding, 1984). Of the 177 psychrotrophic lactics isolated by Hitchener *et al.* (1982) from vacuum-packaged beef, only 18 isolates were identified as *Leuconostoc mesenteroides*. The remainder were lactobacilli, which they classified as "atypical" streptobacteria and betabacteria. Although Hitchener *et al.* (1982) noted considerable variation in the nature of the lactobacilli isolated from vacuum-packaged meat, Egan (1983) suggests that within a vacuum-package there may be relatively few species of lactobacilli and that some packages may even contain a pure culture.

2.2.2 Classification and identification of the "atypical" lactobacilli

In recent years a number of studies have focused on the "atypical" lactobacilli isolated from vacuum-packaged meat, in an effort to understand the groupings that exist among them and the relationship of these organisms to described species (Hitchener *et al.*, 1982; Shaw and Harding, 1984; Holzapfel and Gerber, 1983). Hitchener *et al.* (1982) arranged the atypical lactobacilli into five groups, three

comprised of streptobacteria and two of betabacteria. The atypical streptobacteria could not ferment lactose, maltose or mannitol which is considered typical of *L. plantarum* and is in general also common to all streptobacteria (Sharpe *et al.*, 1966; Hitchener *et al.*, 1982). The streptobacteria were separated into groups based on differences in their sugar fermentation patterns, their ability to hydrolyze esculin and to produce ammonia from arginine in media with low glucose (0.05%) concentration. The betabacteria were atypical in their production of only L(+) lactic acid from glucose as all other described betabacteria produce DL lactic acid (Kandler and Weiss, 1986). The carbohydrate fermentation patterns of the atypical betabacteria also differed from those of described species. Hitchener *et al.* (1982) differentiated the atypical betabacteria into two groups based on differences in their sugar fermentation patterns and also on their ability to produce ammonia from arginine in media with 2.0% (w/v) glucose.

During shelf-life studies on raw, vacuum-packaged as well as SO_2-treated minced beef, 8 atypical betabacteria were isolated by Holzäpfel and Gerber (1983). These strains, like those isolated by Hitchener *et al.* (1982), produced L(+) lactic acid. The atypical betabacteria of this study also differed from typical strains by a low mol% guanine + cytosine (G+C) in the DNA (34 ± 0.8% G+C). As the physiological characteristics of these strains were unlike those of described species, a new species, *L. divergens* was named.

Shaw and Harding (1984) performed a numerical taxonomic study on 100 isolates from refrigerated vacuum-packaged beef, pork, lamb and bacon. Three clusters were observed, all with 78% similarity within clusters. Cluster I was comprised of a group of organisms considered to be atypical streptobacteria, which were referred to as non-aciduric, due to their inability to grow on acetate agar at pH 5.6, to initiate growth at pH 3.9 and in ceasing to grow at a terminal pH > 4.15. The organisms of Cluster I were later (Shaw and Harding, 1985) shown to be heterofermentative. This discrepancy in their description was attributed to medium-dependent gas production by these organisms (Shaw and Harding, 1985). Appreciable quantities of acetate and ethanol produced by glucose breakdown confirmed the heterofermentative nature of these isolates (Shaw and Harding, 1985). The organisms of Cluster II were referred to as aciduric and they were provisionally identified as *Lactobacillus sake* and *L. bulgaricus* (Shaw and Harding, 1984). Cluster III consisted of one strain of *Leuconostoc paramesenteroides* and six unidentifiable *Leuconostoc* strains.

There existed in each of the three studies cited (Hitchener *et al.*, 1982; Holzapfel and Gerber, 1983; Shaw and Harding, 1984) unusual lactobacilli, all betabacteria, which differed in several respects from all other described heterofermentative lactobacilli. To determine the relationship of these atypical lactobacilli, Shaw and Harding (1985) compared representative strains from each study. The strains

could not be separated on the basis of mol% G+C values, although certain properties indicated that the strains consisted of two groups. DNA homology studies revealed that the group was comprised of two very similar species. All the strains in one group showed a high degree of DNA relatedness to the *Lactobacillus divergens* type strain. Strains in the other group were 74-109% homologous with their reference strain. Shaw and Harding (1985) concluded that this latter group represented a new species, closely related to *L. divergens*. The name *Lactobacillus carnis* was proposed for this species, however Shaw (personal communication, 1987) indicated that the lactobacilli described as *L. carnis*, in fact, belong to the previously described species *Lactobacillus piscicola*. *L. piscicola* had not been considered earlier by Shaw and Harding (1984) because it was originally described as a homofermenter which produces DL-lactate. *L.piscicola* can be distinguished from *L. divergens* by the absence of lactobacillic acid in the cellular fatty acids and by its ability to ferment mannitol and α-methyl-glucoside.

2.3 Spoilage of Meat by Lactic Acid Bacteria

The form of spoilage that develops in a food product and how rapidly it develops is dependent upon the composition of the microflora and on the growth rate of the spoilage microorganisms (Gill, 1986). Development of the lactics as the dominant organisms within the vacuum-package

is generally considered desirable (Egan, 1983). Their presence is much less deleterious to the quality of the product than other food spoilage bacteria, such as the pseudomonads. However, the lactics will eventually cause spoilage of the product. In addition to lactic acid, hydrogen peroxide, hydrogen sulfide, acetic acid, ethanol, acetone, 2,3-butanediol or polysaccharides may be produced by the lactics, which may contribute to spoilage (Blickstad, 1983; Shay and Egan, 1981; Sharpe *et al.*, 1983). Spoilage of meat by lactic acid bacteria is due mainly to the flavor defect described by tasters as sour, acid, cheesy and, frequently, as bitter or liver-like (Egan, 1983). Vacuum-packaged beef spoils slowly, long after the lactics have reached their maximum cell density, but in the presence of pure cultures, particularly lactobacilli which produce hydrogen sulfide, the rate of spoilage is increased (Shay and Egan, 1981; Egan, 1983). Among the organisms commonly found on vacuum-packaged meat, the spoilage ability decreases in the following order: *B. thermosphacta* > hetero-fermentative *Lactobacillus* spp. > homofermentative *Lactobacillus* spp. Off-flavor and off-odor are more apparent at an earlier stage with *B. thermosphacta* and hetero-fermentative *Lactobacillus* spp. than with homofermentative *Lactobacillus* spp.

2.4 Control of Microbial Spoilage in Fresh Meats

The shelf-life of aerobically packaged fresh beef is limited by the presence of microorganisms capable of growth at refrigeration temperatures. About 90% of these organisms are gram-negative, and predominatly belong to the *Pseudomonas-Achromobacter* group (Pierson *et al.*, 1970; Reddy and Chen, 1975; Erichsen and Molin, 1981). Spoilage becomes apparent in fresh meat when the glucose source has been depleted. The pseudomonads then attack amino acids, resulting in the formation of malodorous sulfides, esters and acids as by-products (Gill and Newton, 1977; Gill, 1986). Thus, any technique that can retard or inhibit the growth of these microorganisms would improve the shelf-life at refrigeration temperatures.

Various approaches to the control of microbial spoilage in fresh meats have been documented. Gill (1986) has reviewed a number of these approaches including: the addition of glucose to meat; the addition of citrate buffer at pH 4.5 to packs before the vacuum is drawn; and treatment with organic acid, with particular reference to the use of acetic acid. The use of lactic acid as a meat decontaminant was comprehensively reviewed by Smulders *et al.* (1986). Kraft (1986) cited additional procedures for control of psychrotrophs in meat, including the use of hypochlorites and radiation. In this section, research on the use of lactic acid bacteria for retention of fresh meat quality is reviewed.

2.4.1 Use of lactic acid bacteria as a tool for retention of fresh meat quality

Various lactics have been used to extend the shelf-life of aerobically packaged fresh meat (Reddy *et al.*, 1970; Daly *et al.*, 1972; Reddy and Chen, 1975; Gilliland and Speck, 1975; Raccach and Baker, 1978; Abdel-Bar and Harris, 1984). Inoculation of ground beef with *Lactobacillus bulgaricus*, *Lactobacillus lactis* or *Pediococcus cerevisiae* (Gilliland and Speck, 1975) or with *Streptococcus lactis* and *Leuconostoc citrovorum* (Reddy *et al.*, 1970) repressed growth of the gram-negative microorganisms. Daly *et al.* (1972) observed inhibition of *Staphylococcus aureus* by *Streptococcus diacetilactis* in ground beef held at 25°C. Abdel-Bar and Harris (1984) observed significant inhibition of *Pseudomonas fragi*, *Achromobacter liquefaciens* and *S. aureus* in ground beef inoculated with *L. bulgaricus*. In mechanically deboned poultry meat (MDPM), Raccach and Baker (1978) showed that addition of a mixed culture of *P. cerevisiae* and *Lactobacillus plantarum* (Lactacel D3) repressed growth of *Pseudomonas* spp. and prevented growth of *Salmonella typhimurium* and *S. aureus*. In samples incubated at 11°C, *S. typhimurium* increased from 10^3/g to 10^8/g and in samples incubated at 15°C *S. aureus* increased from 10^3/g to 10^7/g in 7 d, but in the presence of the mixed lactic culture there was no increase of either organism. Newton and Gill (1978) studied the interaction of a *Lactobacillus* sp., an *Enterobacter* sp. and *B. thermosphacta* on meat slices.

Although the *Lactobacillus* sp. had the lowest affinity for glucose, its growth was not affected by the presence of the other organisms. At a concentration of $10^7/cm^2$ the *Lactobacillus* sp. inhibited the growth of the *Enterobacter* sp. and *B. thermospacta*.

An inoculum of 10^8 lactics/g, or greater, was required to inhibit growth of the undesirable flora in all studies cited, except one, in which almost complete inhibition was observed with a concentration of 10^6 *L. bulgaricus* cells/g (Abdel-Bar and Harris, 1984). The inhibitory effect of the lactic cultures was shown to be concentration dependent, therefore in some studies the inhibitory effect was improved by increasing the inoculum (Price and Lee, 1970; Reddy *et al.*, 1970; Raccach and Baker, 1978; Abdel-Bar and Harris, 1984). For example, Abdel-Bar and Harris (1984) showed that, when the concentration of *L. bulgaricus* was increased approximately 10-fold, 97.9% of the natural flora of ground beef was inhibited compared with 39.6% inhibition at the lower inoculum level. By increasing the concentration of *L. plantarum* and *P. cerevisiae* 10-fold to 10^9 cells/g, the microflora of MDPM was prevented from attaining 10^7 cells/g (spoilage level) within the 7 d storage period used (Raccach *et al.*, 1979). Whereas, at the lower inoculum concentration of 10^8 cells/g, the shelf-life of the MDPM stored at 3°C, 4 days, was only prolonged to 6 days. Raccach and Baker (1978) showed that the inhibitory effect could also be improved by using a mixture of two lactic strains. The shelf-life of

MDPM was prolonged by 1 d due to treatment with *L. bulgaricus* or *P. cerevisiae* and 2 d with the mixture of both.

Enhancing or improving the inhibitory effect might best be achieved by selecting cultures which have demonstrated superior inhibitory potential, since the inhibitory action of the lactics is not a universal phenomenon. It differs between genera and among strains and may vary from one strain to another in the same species (Dubois *et al.*, 1979). Dubois *et al.* (1979) compared the inhibitory action of different cultures of *Lactobacillus*, *Streptococcus*, *Pediococcus* and *Leuconostoc* by a cross-streaking plate technique. The most inhibitory organisms against 103 bacteria isolated from ground beef were selected. The comparison revealed that cultures from the genus *Streptococcus*, in particular strains of *S. lactis*, were the most inhibitory toward bacteria isolated from ground beef including, *Pseudomonas*, *Serratia* and *Achromobacter* spp. The *Lactobacillus* and *Pediococcus* strains had practically no inhibitory capacity. Dubois *et al.* (1979) suggested that this could account for the fact that Gilliland and Speck (1975) had to use large numbers of *Lactobacillus* and *Pediococcus* to obtain marked inhibition of the fresh beef microflora. Using this argument, Reddy *et al.* (1970) may have required high inoculum levels of *S. lactis* and *L. citrovorum* because the strains selected had poor inhibitory ability. Unfortunately, Dubois *et al.* (1979) did not support their suggestion by demonstrating that strains

with the greatest inhibitory potential on agar were as effective when used to inoculate meat.

Studies on the lactics as biological preservatives in vacuum-packaged meat are in their infancy. Atypical streptobacteria, isolated from vacuum-packaged refrigerated beef were introduced onto beef steaks just before vacuum-packaging (Hanna *et al.*, 1980). By inoculating a high concentration of lactobacilli the counts were numerically higher than the corresponding control at nearly all of the storage intervals tested and consisted of the lactobacilli inoculated. Smith *et al.* (1980), concluded in a companion study that the *Lactobacillus* stains tested by Hanna *et al.* (1980) did not improve storage life or retail case-life and resulted in a markedly decreased palatability of the beef.

2.5 Mechanism of Shelf-life Extension

2.5.1 In aerobically packaged meat

The lactics produce a wide range of inhibitory substances including: organic acids, hydrogen peroxide, antibiotics, and bacteriocins (Hurst, 1973; Raccach and Baker, 1978; Smith and Palumbo, 1981). In general researchers have been content to observe and describe the inhibitory effects of the lactics in fresh meats. As a result the exact mechanism of inhibition resulting in shelf-life extension of these products is unclear (Smith and Palumbo, 1981).

By inoculating the lactics into fresh meat some researchers observed a decrease in product pH (Reddy *et al.*, 1970; Reddy and Chen, 1975; Abdel-Bar and Harris, 1984) while other workers noted no change in pH or a change too small to explain the observed inhibition (Raccach and Baker, 1978; Gilliland and Speck, 1975). Although Reddy *et al.* (1970) offered no explanation for inhibition of the gram-negative microflora in fresh beef, Abdel-Bar and Harris (1984) suggested that organic acids were partly responsible for inhibition. Although the bactericidal and bacteriostatic effects of organic acids are well documented, Gilliland and Newton (1982) found that, of seven different genera isolated from a packing plant, *Pseudomonas* spp. was least adversely affected by the lactic acid concentration in ground beef. Lactic acid may in fact be a promoter rather than an inhibitor of the pseudomonads (Gill, 1976).

Dahiya and Speck (1968), Gilliland and Speck (1975) and Abdel-Bar and Harris (1984) suggested that hydrogen peroxide may be partly responsible for inhibition of certain undesirable bacteria in food. Dahiya and Speck (1968) showed that hydrogen peroxide was the substance inhibitory to *S. aureus* in culture filtrates of *L. lactis* and *L. bulgaricus* and that the concentration of hydrogen peroxide increased for up to 5 days during storage at 5°C. Dahiya and Speck (1968) also showed that a carbohydrate source was necessary for this inhibitory activity by resting cell suspensions of lactobacilli.

Newton and Gill (1978) concluded that inhibition of an *Enterobacter* sp. and *B. thermosphacta* was not due to production of large amounts of acid or to hydrogen peroxide, but was the result of an antibiotic. An inhibitor(s), yet undetermined, which was neither hydrogen peroxide or organic acid, was responsible for inhibition of the gram-negative flora in fresh meat by *P. cerevisiae* (Gilliland and Speck, 1975) and by *P. cerevisiae* and *L. plantarum* (Lactacel DS) (Raccach and Baker, 1978).

2.5.2 In vacuum-packaged meat

The extension in shelf-life of fresh meat achieved by vacuum-packaging can by attributed mainly to failure of the pseudomonads to grow sufficiently (Dainty *et al.*, 1979). With the growth of the pseudomonads restricted, *B. thermosphacta* and *Serratia liquefaciens* become potential spoilage bacteria yet their growth is also restricted by vacuum-packaging (Dainty *et al.*, 1979).

Factors which contribute to the restriction of undesirable bacterial growth in the vacuum-pack include: oxygen deprivation, increased levels of carbon dioxide, and antimicrobial activity of the lactobacilli (Gill, 1986). Carbon dioxide may function by extending the lag phase of the pseudomonads and thus allowing development of the lactics to a point where subsequent growth of the pseudomonads is severely restricted by the synergistic effect of carbon dioxide, low oxygen tension and

antimicrobial agents. Although *B. thermosphacta* and *S. liquefaciens* may be inhibited to some extent by carbon dioxide, this does not fully explain why their growth ceases in vacuum-packed meat. Dainty *et al.* (1979) suggested that antimicrobial activity of the lactics is most likely the cause. Antimicrobial activity of the lactobacilli may explain the failure of the Enterobacteriaceae to increase during prolonged storage of vacuum-packed meat, an increase that would otherwise be expected, as the Enterobacteriaceae can ferment substrates such as glucose-6-phosphate that are present in meat, but not utilized by the lactobacilli.

2.6 Production of Bacteriocins by Gram-Positive Bacteria

Until recently most studies on bacteriocins have centered on those produced by gram-negative bacteria, in particular the prototype bacteriocins, the colicins. Although there has been an increasing number of publications on bacteriocin or bacteriocin-like antagonism by gram-positive bacteria, knowledge in this area remains limited.

Research on bacteriocins produced by gram-positive bacteria is complicated by the fact that there is no universally accepted definition for this group of substances (Tagg *et al.*, 1976). There is a wide range of inhibitory products which may inappropriately be attributed to the action of bacteriocins including: "classical" low molecular weight antibiotics, metabolic products, defective bacteriophage, lytic agents and enzymes. The divisions

between these inhibitors are not clear. As a result a number of often poorly characterized bacterial inhibitors have been somewhat loosely referred to as "bacteriocins".

The following are definitions of bacteriocins given by two groups of researchers:

"Bacteriocins are antagonistic proteins or peptides that show bactericidal activity against closely related species" (Barefoot and Klaenhammer, 1984);

"Bacteriocins are antibiotic-like substances synthesized by certain strains of bacteria against closely related species" (Upreti and Hinsdill, 1975).

In both definitions the bacteriocins were defined based on colicin characteristics (classical criteria). The following classical criteria have been used in varying combinations and applied with different degrees of consistency and proof in defining the bacteriocins of gram-positive bacteria:

(i) a narrow inhibitory spectrum of activity centered around homologous species;

(ii) the presence of an essential, biologically active protein moiety;

(iii) a bactericidal mode of action;

(iv) attachment to specific cell receptors;

(v) plasmid-borne genetic determinants of bacteriocin production and of host cell immunity; and

(vi) production by lethal biosynthesis (Tagg *et al.*, 1976).

The suitability of using these characteristics to define bacteriocin-like substances produced by gram-positive bacteria is questionable since a number of these compounds show discrepancies from the classical criteria. Some of the commonly reported atypical features include: a less solid host cell immunity, and a wider spectrum of activity against gram-positive organisms of different species and even genera. Tagg *et al.* (1976) also cited gram-positive bacteriocins that are active against gram-negative organisms. Exceptions have also been noted to criteria (iv) and (v) (Upreti and Hinsdill, 1975; Joerger and Klaenhammer, 1986).

In the definition given by Upreti and Hinsdill (1975), bacteriocins were referred to as antibiotic-like substances. The narrow inhibitory spectrum and proteinaceous character of the bacteriocins were considered to distinguish these two groups of similar compounds. An antibiotic is usually active against diverse organisms taxonomically distant from that which produces it. However, the boundary between antibiotics and bacteriocins becomes increasingly vague, as some inhibitors with a broad spectrum of activity are considered to be bacteriocins, while others, although proteinaceous in nature, are considered to be antibiotics, such as nisin (Lueck, 1980).

Most of the information regarding the mode of action of bacteriocins has also been based on studies of colicins. A single bacteriocin molecule appears to be sufficient to kill

a sensitive cell (Tagg *et al.*, 1976). The mode of action of a bacteriocin is believed to occur in two stages. In the first stage the bacteriocin absorbs to a receptor on the bacterial cell wall. Generally there is a specific receptor, however non-specific adsorption has been shown in the case of Lactocin 27 (Upreti and Hinsdill, 1975). Removal of the bacteriocin during the first stage by treatment with trypsin, for example, leaves the cell unaffected. In the second stage the cell-bound bacteriocin becomes insensitive to proteases, and the cells undergo irreversible changes. The bacteriocin may interfere with energy metabolism, protein or nucleic acid synthesis, or membrane permeability. Lactocin 27 and Lactostrepcin 5 are bacteriocins produced by the lactics for which the mechanism of action has been elucidated; they interfere with the permeability of the cell envelop (Upreti and Hinsdill, 1975; Zajdel *et al.*, 1985).

Tagg *et al.* (1976) prepared a comprehensive review of the bacteriocins of gram-positive bacteria and discussed the problems that can confront investigators in this field. The literature reviewed in the following section has been selected to illustrate the potential of the lactic acid bacteria, primarly the lactobacilli, to produce substances regarded as bacteriocins and also to illustrate the diverse nature of these substances.

Only a few bacteriocins produced by the lactobacilli have been extensively studied. These include: a bacteriocin produced by a *Lactobacillus fermenti* strain (DeKlerk and

Smit, 1967); Lactocin 27, a bacteriocin produced by *Lactobacillus helveticus* strain LP27 (Upreti and Hinsdill 1973, 1975); Lactacin B, a bacteriocin produced by *Lactobacillus acidophilus* N2 (Barefoot and Klaenhammer, 1984) and Helveticin J, a bacteriocin produced by *Lactobacillus helveticus* 481 (Joerger and Klaenhammer, 1986). These bacteriocins meet many of the classical criteria. All have a narrow spectrum of activity which is restricted to organisms of the genus *Lactobacillus* and have a biologically active protein moiety. The bacteriocin of *L. fermenti*, Helveticin J, and Lactacin B have a bactericidal mode of action, however Lactocin 27 is an anomalous bacteriocin-like agent in that it is only bacteriostatic in its action against sensitive indicator organisms (Upreti and Hinsdill, 1975; Tagg *et al.*, 1976). A plasmid-borne genetic determinant could not be detected for any of these bacteriocins.

In contrast to the bacteriocins described above, other lactics produce bacteriocins which have a broad spectrum of activity (Tagg *et al.*, 1976; Daeschel and Klaenhammer, 1985). For example Pediocin A, produced by a strain of *Pediococcus pentosaceus*, inhibits species of *Pediococcus*, *Lactobacillus*, *Leuconostoc*, *Streptococcus*, *Micrococcus*, *Staphylococcus*, *Bacillus* and *Clostridium*, but was not active against the gram-negative bacteria tested (Daeschel and Klaenhammer, 1985). Pediocin A is proteinaceous and has a bactericidal mode of action. Both the genetic determinants

for production of Pediocin A and host immunity are encoded by a 13.6 megadalton plasmid.

The bacteriocins, discussed above, show the diverse nature of this class of antimicrobial compounds produced by the lactics. Some bacteriocins (Helveticin J, Pediocin A, Lactacin B) inhibit only closely-related species or inhibit species within a number of gram-positive genera (Pediocin A); some are bacteriostatic in their activity (Lactocin 27) or bactericidal (Lactacin B); some are encoded by genetic determinants that reside on plasmid DNA (Pediocin A) or have chromosomally-borne genetic determinants (Helveticin J), but in all cases they differ slightly from the classical colicin model.

3. MATERIALS AND METHODS

3.1 Bacterial Cultures

The lactic cultures used in this study are listed in Table 3.1. They were obtained from the American Type Culture Collection (ATCC), kindly donated by the indicated sources (Table 3.1) or isolated from vacuum-packaged beef. Isolates #1-#61 were randomly selected from vacuum-packaged beef samples plated onto APT agar (Difco Laboratories, Detroit, MI) and Lactobacilli MRS agar (deMan, Rogosa and Sharpe, 1960) adjusted to pH 5.5 (Baird and Patterson, 1980). Isolates A-P were collected in a previous study (Harris, 1986) from irradiated vacuum-packaged meat samples plated onto MRS agar incubated at 20°C. All lactic acid bacteria used in this study were shown to be gram-positive, and catalase- and benzidine-negative. Non-lactic indicator cultures were obtained from ATCC and other sources as indicated in Table 3.2.

3.2 Media

The culture media used in this study are listed in Table 3.3. Media were prepared as directed by the manufacturer or as described in the references cited. Agar (0.75% w/v) was added to APT broth or to trypticase soy broth (TSB) to make soft (overlay) agars for lactic and non-lactic indicators, respectively. The pH of $MRS_{(5.5)}$ agar was adjusted to 5.5 with lactic acid (85%). pH adjustment of

Table 3.1. The lactic acid bacteria used in this study and their sources.

Organism	Source[a]
Pediococcus pentosaceus[1]	Lee[b]
Pediococcus cerevisiae (Hansen)	Lee
•*Pediococcus cerevisiae* (Lactacel)	Lee
•*Pediococcus acidilactici*	ATCC 8042
•*Pediococcus parvulus*	ATCC 1937
C 4[2]	Holley[c]
•C 35[2]	Holley
C 43[2]	Holley
•*Lactobacillus viridescens*	ATCC 12706
•*Lactobacillus plantarum*	ATCC 4008
Lactobacillus plantarum (Rosellac C)[3]	Holley
•*Lactobacillus R.S.M.*[4]	Holley
•*Leuconostoc mesenteroides*	ATCC 23368
Meat Isolates #1-61	V-P beef
Meat Isolates A-P	Irradiated V-P beef

[a] ATCC = American Type Culture Collection; V-P = vacuum-packaged

[b] Dr. B. Lee, Agriculture Canada, Ste. Hyacynthe, P.Q

[c] Dr. R.A. Holley, Food Research Institute, Agriculture Canada, Ottawa, Ont.

[1] isolate from commercial starter Lactacel 75, Lot 09212

[2] isolates from commercially cured meats, cooked or uncooked salami

[3] isolate from Roselle meat starter culture Lot 27 B.E.

[4] isolate from santamaria italian salami

• Used as indicator organisms for initialscreening of test cultures

Table 3.2. The non-lactic indicator organisms used in this study and their sources.

Organism	Source
Gram-Positive:	
Bacillus cereus	NCDO 577
Bacillus cereus	ATCC 14579
Clostridium perfringens	JACKSON[1]
•*Streptococcus faecalis*	ATCC 7080
Streptococcus bovis	ATCC 15351
Listeria monocytogenes	ATCC 15313
Listeria monocytogenes	HPB 81-861[2]
Listeria monocytogenes	HPB 85-307[2]
Listeria innocua	ATCC 33090
Staphylococcus aureus	ATCC 25923
Staphylococcus epidermidis	ATCC 12228
Gram-Negative:	
•*Escherichia coli*	ATCC 1840
•*Klebsiella pneumoniae #2*	STILES[3]
Klebsiella pneumoniae	ATCC 13883
Klebsiella oxytoca	STILES[3]
•*Serratia liquefaciens*	ATCC 27952
•*Yersinia enterocolitica*	ATCC 23715
•*Salmonella typhimurium*	ATCC 13311
•*Alteromonas putrefaciens*	ATCC 8071
•*Pseudomonas aeruginosa*	ATCC 27853

[1] Dr. H. Jackson, Department of Food Science, University of Alberta

[2] Isolates from the Health Protection Branch, Health and Welfare Canada, Ottawa, Ont.

[3] Stiles and Ng (1980)

• Indicator organisms used for initial screening of test cultures

Table 3.3. The culture and selective plating media used in this study and their suppliers or reference.

Name	Supplier or Reference
Culture Media:	
APT Broth/Agar	APT, Difco[1]
Cooked Meat Medium	CMM, Difco
Trypticase Soy Broth/Agar	TSB, TSA, BBL[2]
Basal Medium	BM, Wilkinson and Jones (1977)[3]
SBM	SBM, Wilkinson and Jones (1977)[3]
Selective Plating Media:	
Acetate Agar	AA (5.6), Rogosa *et al.* 1951[3]
MRS Agar (pH 5.5)	MRS (5.5), Difco
MRS Agar (pH 5.6)	MRS (5.6), Difco
Lactate Agar	LA, Shaw and Harding (1984)[3]
Streptococcus KF agar	KF, Difco

[1] Difco = Difco media, Difco Laboratories, Detroit, MI

[2] BBL = Becton Dickinson and Co., Cockeysville, MD

[3] Prepared from Difco ingredients and chemicals from Fisher Scientific or J.T. Baker Scientific

all other media was done with 1.0 N NaOH or 0.1 N HCl. The composition of lactobacilli MRS medium (Difco) and modifications of MRS media used in this study are listed in Table 3.4 (see p. 46). Modified MRS broth or agar, referred to as BM broth or agar by Wilkinson and Jones (1977), was used as a general culture medium and also as a basal medium for a number of biochemical tests.

3.3 Conditions for Growth and Maintenance of Stock Cultures

Lactic cultures were maintained in Cooked Meat Medium (CMM) at 4°C and subcultured every three months. Before use, the cultures were propagated twice in APT or BM broth for 18-24 h at 25°C. Non-lactic cultures were maintained on tryptic soy agar (TSA) slants at 4°C and subcultured monthly. These cultures were subcultured twice in APT at 25°C (lactics) or tryptic soy broth (TSB) at 35°C (non-lactics), before use in experimental studies.

3.4 Isolation of Lactic Acid Bacteria from Vacuum-Packaged Beef

Lean ground beef, purchased from a local supermarket, was divided into approximately 100 g portions and vacuum-packed in tri-layer "Vacpac" bags (Cryovac, DRG Packaging, Edmonton, Alberta; O_2 transmission 30-50 $cm^3/m^2/h$, 22°C, 1 atm.). These samples were stored at 4°C for up to 8 weeks. After 6 or 8 weeks, 10 g samples were homogenized with 90 mL of sterile 0.1% peptone water (Difco) for 1 min in a

Colworth Stomacher (Model 400, A.J. Seward, Sulfolk, U.K.). Serial dilutions of the homogenate were prepared in 0.1% peptone water, and suitable dilutions were surface plated in duplicate onto both $MRS_{(5.5)}$ and APT. One set of plates was incubated aerobically at 25°C for 2 days and the other was incubated anaerobically at 4°C for 7 days. Anaerobic conditions were created by flushing an anaerobic jar with a gaseous mixture of 95% H_2 and 5% CO_2. Anaerobiosis was verified with a Gas Pak Anaerobic Indicator (BBL Becton, Dickinson & Co., Cockeysville, MD). Typical colonies were randomly selected from the $MRS_{(5.5)}$ and APT agar plates. Each isolate was propagated twice in APT broth at 25°C overnight before characterization (gram stain, benzidine and catalase tests) and storage in cooked meat medium.

3.5 Development of Screening Procedures for Bacteriocin-like Antagonism

A number of direct antagonism procedures were investigated, including the methods of Tagg *et al.* (1973), Geis *et al.* (1983) and Barefoot and Klaenhammer (1983). In all methods the plates were incubated at 25°C.

For the deferred antagonism technique a number of procedures was investigated to find a suitable treatment for killing the bacterial cells (Tagg *et al.*, 1973; Upreti and Hinsdill, 1973; Geis *et al.*, 1983). Culture plates with and without the surface growth removed were exposed to chloroform vapor for 30, 60, 90 and 120 min by placing the plates

in a sealed container with chloroform or by inverting the culture plates over glass petri dishes containing a few drops of chloroform. Culture plates were exposed to UV light in a UV hood (Labconco Corporation, Kansas City, MO) for 30, 60 and 120 min. In some trials, following the UV treatment, the culture plates were also exposed to chloroform vapor for 30 and 60 min. Additionally, some culture plates were placed in a drying oven at 70°C for 30, 60 and 90 min. To determine the lethal effect of a particular treatment, treated agar plates or tubes of APT broth, inoculated with colonies removed from treated plates, were incubated overnight at 25°C. Visible growth following incubation at 25°C indicated that some cells had survived the treatment. The methods of deferred and direct antagonism adapted for use in this study are as described below.

3.6 Screening for Bacteriocin-like Antagonism

Each of the lactic cultures listed in Table 3.1 was tested for its ability to inhibit a number of indicator strains by deferred and direct (simultaneous inoculation) antagonism procedures (Tagg *et al.*, 1976). To detect the simultaneous antagonism of one strain (indicator strain) by another (test strain), the test strain was inoculated onto the surface of an APT agar plate using a replicating inoculator (Cathra International Systems for the Microbioligist, Diagnostic Equipment, Inc., St. Paul, MN). A maximum of 4-5 test strains was inoculated per plate,

following which the surface of the plates was dried at 25°C for 30-40 min in a Bioflow chamber (Germfree Laboratories, Inc., Miami, FL). After drying, an overlay, containing approximately 10^6 CFU of the indicator culture/mL, was poured onto the surface of each plate. Each indicator overlay was prepared by adding 0.15 mL from a 10^{-1} dilution of an 18 h indicator culture to 4.5 mL of APT soft agar, and gently mixing. All indicator overlays were prepared in this manner, unless otherwise stated. The plates were then incubated anaerobically overnight at 25°C. Failure of the indicator strain to grow in the vicinity of the test strain indicated the release of growth inhibitor(s) by the test strain.

In the deferred procedure, the test strain was inoculated onto APT plates and incubated anaerobically for 18-24 h. After pre-incubation of the culture plates, the surface growth was removed with a glass slide. Any remaining bacterial cells were killed by placing the plates above the water level in a covered 80°C water bath for 30 min. Following this heat treatment, the surface of the agar plates was dried for 30-40 min in the sterile Bioflow chamber before adding the indicator overlay. The plates were incubated anaerobically overnight at 25°C and examined for zones of inhibition in the indicator lawns.

Using these screening procedures, only one isolate, strain #26, from vacuum-packed meat was found to be antagonistic to the indicator cultures tested (refer to

Results p. 58). Because of the relatively broad spectrum of antimicrobial activity demonstrated by this isolate, it was used for further study.

3.7 Tests to Distinguish Antagonism Unrelated to Bacteriocin Production

Several factors can mimic bacteriocin activity in the deferred and direct screening tests (Tagg *et al.*, 1976). Tests were performed to determine whether the observed inhibition was caused by the release of bacteriophage, production of hydrogen peroxide, or development of organic acids.

3.7.1 Bacteriophage infectivity testing

The method outlined by Gangliano and Hinsdill (1970) was used. Following the deferred antagonism test, a block of agar was aseptically removed from an area within a zone of inhibition, and crushed in a glass tube with a sterile metal spatula. Sterile APT broth (10 mL) was added to these tubes prior to incubation overnight at 25°C. From samples which showed no bacterial growth, a 0.1 mL aliquot was added to a soft APT agar overlay containing an indicator culture, gently mixed, and poured onto the surface of an APT agar plate. Following incubation, the culture plates were examined for plaques indicating the presence of phage.

The reverse-side agar technique described by Scherwitz *et al.* (1983) was also used to determine if the observed

antagonism was due to bacteriophage. In this procedure, test cultures were grown anaerobically for 18 h on APT agar plates. Following incubation, the agar was detached from the edges of the petri dish using a sterile spatula. The covered plate was then inverted and tapped on a hard surface so that the agar fell into the lid and an indicator overlay was poured onto the inverted agar. Following anaerobic incubation for 24 h at 25°C the plates were examined for zones of inhibition, indicating the ability of the inhibitor to diffuse through the agar.

3.7.2 Hydrogen peroxide production

Two lines of evidence were used to eliminate hydrogen peroxide as the bacterial inhibitor. Throughout the study, cell culture plates were incubated anaerobically. As a second line of evidence, in some trials, the APT agar and APT soft agar overlay were supplemented with 68 units of filter-sterilized catalase (Sigma Chemical Co., St. Louis, MO) per mL of agar or soft agar (Barefoot and Klaenhammer, 1983). Production of the inhibitor during anaerobic incubation or in the presence of catalase shows that the inhibitor is not hydrogen peroxide.

3.7.3 Neutralization of acidity

To determine whether acidity was responsible for the observed antagonism, the method of Dahiya and Speck (1968) was modified for use in this study. APT agar (100 mL) was

tempered to 45°C and mixed with 10 mL of a 20% calcium carbonate solution. Addition of calcium carbonate to the agar makes it impossible to detect growth of the test culture; therefore, 1 mL (0.01% w/v) of 2,3,5-triphenyl-tetrazolium chloride (TTC) was added to the agar-calcium carbonate mixture. Growth of isolate #26 was detected by its ability to reduce TTC which turns the agar red. This modified APT agar was added in 10 mL aliquots to petri dishes to prepare 5 uninoculated control plates. The remainder of the agar mixture was inoculated with 0.1 mL of an 18 h test culture and similarly dispensed into sterile petri dishes. Wells were formed in the solidified agar by pressing a sterile metal cap (diameter 22 mm) onto the surface to form two circles, and then removing the agar within these circles using a sterile spatula. A tube containing 10 mL of tempered soft APT agar, and 0.1 mL of a 10^{-1} dilution from an indicator culture was mixed. Approximately 1 mL amounts of this mixture were used to fill one circle in both the control and inoculated APT agar plates. The other circle of each plate was filled with soft agar containing a second indicator culture. Following anaerobic incubation at 25°C for 24 h, the plates were examined for inhibition of the indicator cultures.

3.8 Conditions Affecting Inhibitor Production on Solid Media

3.8.1 Effect of pH

APT agar, dispensed in 25 mL amounts into screw cap tubes, was adjusted to pH 5.0, 5.5, 6.0, 6.5, 7.0 and 7.5 with 1 N HCl and 85% lactic acid or with 1 N NaOH, and poured into petri dishes. The pH of the poured agar plate was confirmed using an Orion combination pH electrode (Orion Research, Inc., Cambridge, MA) and also with pH paper strips (EM Universalindikator pH 0-14). The pH-adjusted plates were compared as substrates for production of growth inhibitor(s) by the deferred antagonism technique.

3.8.2 Effect of incubation temperature

Sets of APT plates were inoculated and incubated anaerobically at 1, 4 and 10°C in low temperature incubators for up to 8 d. After various incubation periods, the deferred inhibition procedure was performed on 2 plates from each temperature.

3.9 Sensitivity to Proteolytic Enzymes

The method described by Scherwitz *et al.* (1983) was modified for use in this study to assess the susceptibility of the inhibitory substance(s) to a proteolytic enzyme. Following the deferred antagonism technique, 0.4 mL of protease No. P-5380 (Sigma Chemicals Co., St. Louis, MO; 1 mg/mL in 0.1 M sodium phosphate buffer, pH 7.0) was added to

the agar plate. The plate was then overlayered with an indicator culture and incubated at 25°C overnight. No zone of inhibition compared with inhibition on control plates treated with buffer only or heat inactivated enzyme indicates sensitivity to the proteolytic enzyme.

3.10 Partial Purification of the Inhibitor(s)

Partial purification of Bac #26 was attempted by the method of Geis *et al.* (1983). Following overnight incubation in APT broth (100 mL), the cells of isolate #26 were removed by centrifugation (3,000 x g for 20 min). The neutralized supernate was concentrated to 1/10 its original volume with a rotary evaporator (Buchler Instruments, Fort Lee, N.J.) at 40°C. Solid ammonium sulfate was added at 100 % saturation to the concentrated supernate and gently stirred overnight at 4°C. The ammonium sulfate precipitate was sedimented by ultracentrifugation (50,000 x g for 1 hr). Following centrifugation, the precipitate was resuspended in a minimal amount of tris buffer (10mM, pH 8.0) and extensively dialyzed against the same buffer overnight. The dialyzed solution was tested for inhibitory activity (see below).

Partial purification of Bac #26 was also attempted by preparing culture extracts. Culture extracts were prepared using a modification of the method of Barefoot and Klaenhammer (1983). A 0.1 mL aliquot of an 18 h APT broth culture was surface inoculated onto each of seven APT agar plates. After anaerobic incubation for 72 h, the agar was removed

from the petri dish, weighed, and added to an equal weight of sterile phosphate buffer (3 mM NaH_2PO_4-Na_2HPO_4, pH 7.0) in a Whirl-Pak bag ("Nasco", Systems Plus, Waterloo, Ont.). The buffer-agar mixture was crushed by hand and allowed to equilibrate for 24 h at 4°C, following which the mixture was pre-filtered through Whatman no. 1 filter paper (Whatman Ltd., Kent, England). After centrifugation at 10,000 x g for 10 min, the supernatant was concentrated 20-fold in an Amicon (Model 8400, Amicon Canada Ltd., Oakville, Ont.) under N_2 pressure containing a diaflow ultrafiltration membrane (PMIQ, Amicon). The retentate (pH 5.1) was then heated for 30 min at 60°C and/or adjusted to pH 6.5 or 7.0 with 3 N NaOH.

Inhibitory activity of the culture extracts was tested using the modification of Tagg and McGiven's method described by Barefoot and Klaenhammer (1983). Wells were cut aseptically in APT agar plates and sealed by the addition of two drops of sterile agar. Culture extracts (50 µL), or as a control 50 µL of sterile 3 mM phosphate buffer, were placed in sealed wells and allowed to diffuse into the agar for 6 h at 25°C. Following diffusion, an indicator overlay was poured over the surface of the APT well plate.

3.11 Physiological, Metabolic and Biochemical Tests

Isolate #26 was examined morphologically by preparing a gram stain of an 18 h culture grown in APT broth.

3.11.1 Physiological tests

(a) Growth range. The temperature range of growth for isolate #26 was estimated in a temperature gradient incubator (Scientific Industries Inc., New York). Culture tubes containing 10 mL of APT broth were allowed to equilibrate overnight in the incubator prior to inoculation with 0.1 mL of an 18 h culture diluted 100-fold in 0.1% peptone water. The tubes were shaken at approximately 50 strokes per min. Temperature readings across the gradient were taken daily in a duplicate interspaced set of tubes, containing water, using a probe thermometer (Caspar Integrated Systems, Fort Bragg, CA). Growth was determined by measurement of OD_{600} mμ at 24 h intervals for up to 14 days using a Spectronic 20 spectrophotometer (Bausch & Lomb, Rochester, NY).

(b) Growth at various temperatures. Tubes of BM broth inoculated with a 1% inoculum of isolate #26 were incubated in refrigerated incubators at 1 and 4°C for 8 days, at 15°C and in a heated water bath at 45°C for 5 days and examined for visible signs of growth (Hastings and Holzapfel, 1987).

(c) Growth at pH 3.9 and 4.4. Tubes of modified APT broth (with phosphate omitted) adjusted to pH 3.9 and 4.4 with 1 N HCl (Shaw and Harding, 1984) were inoculated with isolate #26 and observed for visible signs of growth after 7 days incubation at 25°C.

(d) Growth in the presence of inhibitors. The methods and times of incubation described by Wilkinson and Jones

(1977) were used for growth in broth or agar containing one of the following inhibitors (all percentages w/v): 8% NaCl; 0.1% $NaNO_2$; 2.5% potassium thiocyanate; 0.5% potassium tellurite; 0.01% 2,3,5-triphenyltetrazolium chloride (TTC).

(e) Growth on acetate or lactate agars. Cultures were streaked onto $acetate_{(5.6)}$ and $lactate_{(5.6)}$ agar, incubated anaerobically for 5 days, and examined for visible signs of growth (Shaw and Harding, 1984).

(f) Survival at 60°C for 15 min. Overnight cultures in BM broth were placed in a water bath at 60°C for 15 min. A pasteur pipette was used to place 2 drops of the heated culture onto BM plates. The plates were incubated at 25°C for 3 days and observed for visible signs of growth (Hastings and Holzapfel, 1987).

3.11.2 Metabolic and biochemical characteristics

(a) Fermentation tests. The following carbon sources were tested using the laboratory procedure outlined by Klaenhammer (personal communication): amygdalin, arabinose, cellobiose, dextrose, galactose, potassium gluconate, glycerol, lactose, maltose, mannitol, melezitose, melibiose, raffinose, rhamnose, ribose, salicin, sorbitol, sucrose, trehalose and xylose. All carbon sources were prepared as 4% stock solutions and added in 0.05 mL amounts to the wells in a sterile Minitek plate (BBL, Becton Dickinson and Co., Cockeysville, MD). The test culture was prepared by transferring enough growth from an overnight MRS culture

plate with a sterile swab to 5 mL double strength MRS identification broth (2X MRS I.D.; see Table 3.4) to give an optical density approximating a number 5 McFarland standard. A 0.05 mL aliquot of this culture suspension was added to each of the wells containing the test solutions.
As controls, 2X MRS I.D. broth (0.05 mL) was added to a second set of wells containing each test solution (0.05 mL) and a well containing distilled water (0.05 mL). A third well containing the culture suspension and distilled water (0.05 mL of each) was also prepared as a control.

After anaerobic incubation of the inoculated Minitek plates for 2 days at 25°C, 0.05 mL of bromocresol purple (0.04% w/v) was added directly to each well. The wells were then scored as positive by development of a bright yellow color, as (±) by development of a yellow-purple or yellow-gray color, and as negative, if the indicator remained purple or turned gray.

(b) Production of ammonia from arginine. The method described by Klaenhammer (personal communication) was used. The procedure outlined in (a) above was followed, except that the culture suspension was added to a well containing 0.05 mL of an arginine stock solution (arginine·HCl, 2% w/v and glucose 2% w/v). After the addition of bromocresol purple, development of a purple color indicated a positive result, and a yellow color indicated a negative result.

(c) Detection of esculin hydrolysis. The method described by Klaenhammer (personal communication) was used.

Table 3.4. The composition of MRS and modified MRS media.

Composition (g/L)	Media[1]					
	MRS agar	BM medium	2X MRS/I.D.	GP agar	LI broth	La broth
Peptone	10	10	20	10	10	10
Beef extract	10	10	--	10	--	10
Yeast extract	5	5	10	5	5	5
Dextrose	20	20	--	20	2%[3]	20
Tween 80 (mL)	1	1	2	1	1	1
K_2HPO_4	2	2	--	2	2	--
$Na_2HPO_4 \cdot 7H_2O$	--	--	4	--	--	--
Sodium acetate	5	--	10	5	--	5
$(NH_4)_3$ citrate	2	--	--	--	2	--
$(NH_4)_2H$ citrate	--	--	4	--	--	--
Sodium citrate	--	--	--	--	--	3
$(NH_4)_2SO_4$	--	--	--	2	--	--
$MgSO_4 \cdot 7H_2O$	0.2	0.2	0.2[2]	0.2	0.2	0.2
$MnSO_4 \cdot 4H_2O$	0.05	0.05	0.1[2]	0.05	0.05	0.05
Agar	15			15		

[1] Abbreviations:
MRS, Lactobacilli MRS (Difco) as proposed by DeMan, Rogosa and Sharpe (1960)
BM, Basal Medium (Wilkinson and Jones, 1977)
2X MRS/I.D., Double Strength MRS identification broth (Klaenhammer, personal communication)
GP, modified MRS agar used for determination of gas production from glucose (Hitchener *et al.*, 1982)
LI, MRS medium without acetate and beef extract (Shaw and Harding, 1985) used for growth of cultures prior to lactate isomer determination
La broth (Shaw and Harding, 1984)

[2] Added in the form of a salt solution (2 mL/L): $MgSO_4$, 10 g; $MnSO_4$, 5 g; distilled H_2O, 100 mL

[3] Glucose added as a filter-sterilized solution

The procedure described in (a) above was followed, except that the culture suspension was added to a well containing 0.05 mL of the esculin stock solution (esculin 4% w/v and ferric ammonium citrate, brown granular, 1% w/v). Following incubation, development of a black color indicated a positive reaction and a brown color indicated a negative reaction.

(d) Reduction of potassium tellurite and 2,3,5-triphenyltetrazolium chloride (TTC). Test cultures were streak inoculated onto BM agar containing 0.05% (w/v) potassium tellurite and onto SBM agar containing 0.01% and 0.1% (w/v) TTC (see Table 3.3). Blackening of the agar or the formation of a deep red color after 1 or 2 days incubation indicated reduction of tellurite and TTC, respectively.

(e) Nitrate reduction. The method described by Wilkinson and Jones (1977) was used. Tubes of BM broth plus 0.1% KNO_3 containing an inverted Durham tube were examined after 2 and 8 days incubation at 25°C for the presence of gas. At the same time, samples were removed and tested for the presence of nitrite, as described by Wilson and Miles (1964). A positive result (red color) occurs within 1 to 2 min. Negative tubes (no color development) were tested for the presence of unreduced nitrate at 8 days by adding zinc dust. At this stage, a positive test (no color development) indicates that nitrate was reduced to nitrite and then further reduced to non-gaseous products and a negative test

(pink to deep red color formed within 5 to 10 min) indicates nitrate was not reduced.

(f) Catalase. After anaerobic incubation for 2 days on BM agar, one drop of hydrogen peroxide (30%) was added directly to the test culture on BM agar.

(g) Terminal pH. The pH of the cultures was measured after 7 days incubation at 25°C in La broth (see Table 3.4).

(h) Production of gas from glucose. Production of gas from glucose was determined by the method of Gibson and Abdel-Malek (1945) using the modification described by Hitchener *et al.* (1982). Screw cap tubes were filled to a depth of 5-6 cm with modified MRS agar referred to as GP agar (see Table 3.4). Following sterilization, GP agar was cooled to 45°C and inoculated with a 0.1 mL aliquot of an overnight test culture. After the agar had hardened, vaspar tempered to 45°C was poured into the tube to form a layer 2-3 cm deep above the agar stub. The tubes were incubated at 25°C for 10 days and observed daily for gas production, indicated by accumulation of gas under the vaspar plug.

(i) Identification of lactate isomer. The test culture was grown for 2 days in LI broth (see Table 3.4) at 25°C in stoppered glass flasks flushed with a 95% H_2 and 5% CO_2 gas mixture. The cells were removed by centrifugation at 8,000 x g for 10 min. L- and D-lactate were determined enzymatically from the test culture supernatant using the reaction kits obtained from Boehringer (139084). In this procedure, L- and D-lactic acid are converted to pyruvate

using stereospecific L- or D-lactate dehydrogenases (LDH) in the presence of nicotinamide-adenine dinucleotide, NAD (Equation 1) (Food Analysis, Boehringer Mannheim).

Equation 1:

$$\text{L-lactate} + \text{NAD}^{+} \xrightleftharpoons{\text{L-LDH or D-LDH}} \text{pyruvate} + \text{NADH} + \text{H}^{+}$$

The reaction equilibrium lies almost completely on the side of lactate, so it is necessary to trap the pyruvate to drive the reaction to completion. The equilibrium can be displaced in favor of pyruvate and NADH in a subsequent reaction catalyzed by glutamate-pyruvate transaminase (GPT) in the presence of L-glutamate (Equation 2) (Food Analysis, Boehringer Mannheim).

Equation 2:

$$\text{pyruvate} + \text{L-glutamate} \xrightleftharpoons{\text{GPT}} \text{L-alanine} + \alpha\text{-ketoglutarate}$$

The amount of NADH formed in the above reaction is stoichiometric with the concentration of L-(+)- or D-(-)-lactic acid present in the supernatant. The increase in NADH was measured by absorbance at 340 nm using a Hewlett Packard 8451A Diode Array Spectrophotometer. The concentration of each lactate isomer was calculated using the following equation:

$$c = \frac{V \times MW}{\epsilon \times d \times v \times 100} \times \Delta A \ [g/L]$$

where: V = final volume; MW = molecular weight of the substance to be assayed; ϵ = absorption coefficient of NADH; d = light path (cm); v = sample volume; ΔA = absorbance difference = ΔA_S (sample) - ΔA_B (blank) (Food Analysis Boehringer Mannheim). Standard solutions containing known concentrations of the L- or D-isomer of lactic acid were prepared as controls.

(j) Methyl red test. A positive methyl red test was indicated by the formation of a bright red color after addition of a few drops of methyl red solution to cultures grown in MRVP broth (Difco).

(k) Pseudocatalase. Isolate #26 was grown on tryptose agar containing 5% sheep blood for 48 h. A positive result was indicated by bubbling of the hydrogen peroxide (30%) dropped onto the bacterial growth (Hastings and Holzapfel, 1987).

(l) Oxidative/fermentative utilization of glucose. The method of Hugh and Leifson (1953) was used. Test cultures were stabbed into two tubes of OF Basal medium (Difco) containing 1% (w/v) glucose. One tube of each set was overlayered with approximately 1.0 to 2.0 mL of sterile vaspar. Culture tubes were incubated at 25°C and examined at frequent intervals for up to 14 days. Fermentative capacity is indicated by yellow (acid production) in both the open and covered tubes.

(m) An API 20S™ Streptococcus System (API Laboratory Products Ltd., St. Laurent, Quebec) was used to determine if

β-glucosidase, N-acetyl-glucosaminidase, β-galactosidase, phosphatase, leucine and serine aminopeptidase, pyroglutamic acid, acylamidase, and/or aminopeptidase are produced. Growth of isolate #26 removed from an APT culture plate was resuspended in sterile 0.85% saline to approximate a No. 1 McFarland standard. Each microcupule was filled with 2-3 drops of the bacterial suspension. Results were recorded after 4 h incubation at 25°C.

3.12 Growth in Ground Beef Samples

3.12.1 Preparation of lean ground beef samples

Fresh, lean ground beef purchased from a local supermarket was packaged in 10 g portions in "Whirl-Pak" bags ("Nasco", Systems Plus, Waterloo, Ont.). These samples were irradiated in a Gammacell 220 (Atomic Energy of Canada Ltd., Ottawa, Ont.) for 24 h, resulting in exposure to approximately 880 krads. A prepared inoculum containing both *S. faecalis* ATCC 7080 and isolate #26 (see below) was added to the irradiated ground beef and mixed thoroughly into the sample by manipulating the meat in the sealed "Whirl-Pak" bag. Additionally, a set of uninoculated samples, a set inoculated with a pure culture of *S. faecalis* ATCC 7080 (10^5 CFU/g), and a set inoculated with only isolate #26 (10^5 CFU/g) was prepared. The meat samples were incubated in anaerobic jars flushed with 95% H_2 and 5% CO_2 at 25°C or at 4°C.

3.12.2 Preparation of inocula for ground beef samples

Either 10 mL or 100 mL of an 18 h culture of isolate #26 was centrifuged at 6,500 x g and 8,000 x g, respectively, for 10 min. The cell pellet was resuspended in 1 mL or 10 mL volumes of sterile 0.1% peptone water to achieve a desired inoculum level. An 18 h culture of *Streptococcus faecalis* ATCC 7080 was diluted 100-fold in 0.1% peptone water. Equal volumes (0.1 mL) of the resuspended isolate #26 cells and the diluted *S. faecalis* culture were mixed immediately prior to inoculation. A 0.2 mL aliquot of these mixtures was used to inoculate each irradiated ground beef sample. Sets of samples were prepared to contain approximately 10^5 of *S. faecalis* and 10^5, 10^7, 10^8, or 10^9 CFU of isolate #26/gram of meat.

3.13 Growth in Nutrient Broth

Nutrient broth (10 mL) in screw cap test tubes was used as a substrate for associative growth of *S. faecalis* and isolate #26 test cultures. Following inoculation (inocula were prepared as described for the meat samples), the samples were mixed by vortex and overlayered with 2 mL of sterile mineral oil (Sigma) to reduce the oxygen tension. Inoculated tubes were stored at 25°C or at 4°C.

3.14 Sample Testing

The meat and broth samples at both temperatures were tested on day 0, immediately after inoculation, and every day or alternate day thereafter for 8 or 9 days. Sterile 0.1% peptone water (90 mL) was added directly to the meat sample in the Whirl-Pak bag and homogenized for 1 min using a Colworth Stomacher. Duplicate APT and Streptococcus KF agar plates were surface inoculated with 0.1 mL of a suitable dilution. APT agar plates were incubated anaerobically for 48 h at 25°C and the KF plates were incubated aerobically for 24 h at 35°C. The APT agar was used to enumerate the total bacterial population, including both isolate #26 and *S. faecalis* cells.

There was no media found that could selectively enumerate isolate #26. Although *S. faecalis* and isolate #26 could not be distinguished morphologically after 48 h on APT agar, an additional overnight incubation period (aerobically, 25°C) made differentiation possible due to autolysis of isolate #26 colonies (see Results p. 64). Therefore, a total count of the sample was determined at 48 h and an isolate #26 count at 72 h. *S. faecalis* bacterial counts were determined on KF agar.

3.15 pH Measurement of the Meat Samples

After homogenization in 0.1% peptone water and microbial analysis of the meat sample, the pH was measured using an Orion combination pH electrode (Orion Research

Inc., Cambridge, MA).

3.16 Plasmid Analysis

The technique used to obtain plasmid DNA from isolate #26 was described by Klaenhammer (1984). For cell lysis, 75 µL mutanolysin and/or lysozyme (1 mg/mL in 50 mM Tris-HCl, 5 mM Na_2EDTA, pH 7.5) was added to the sample held in an ice bath for 1 h or, in some trials, to the sample at 37°C for 1 h. Agarose gel electrophoresis was done as described by Klaenhammer (1984) using sample volumes of 20 µL.

4. RESULTS

4.1 Development of Screening Procedures

Methods of detecting bacterial antagonism, referred to as direct or deferred antagonism procedures (Tagg *et al.*, 1976) were developed for use in this study. The direct procedure used in this study is similar to the one described by Geis *et al.* (1983).

A number of treatments for killing bacterial cells was examined for use in the deferred procedure. Bacterial cells survived at various time intervals of exposure to chloroform vapor, UV light and dry heat. The 30 min heat treatment, in the covered water bath set at 80°C, was the only treatment in which visible growth did not occur during subsequent incubation, therefore, this treatment was used.

4.2 Incidence of Inhibitor Production

Lactic cultures, primarily isolates from vacuum-packaged beef, were initially screened for their ability to inhibit the growth of 16 arbitrarily selected lactic and non-lactic indicator cultures (indicated by a "•" in Tables 3.1 and 3.2). Of nearly 100 isolates screened, only four test cultures, *Pediococcus cerevisiae* (Hansen), *Lactobacillus plantarum* (Rosellac C), C 4, and an isolate from vacuum-packaged meat referred to as isolate #26, produced substances antagonistic to the growth of the selected indicator cultures on APT. Inhibitor(s) production was

evident in the direct procedure by zones of inhibition 3-5 mm in diameter surrounding the producer strains, and in the deferred procedure by zones 10-20 mm in diameter in lawns of the sensitive indicator culture (Figure 4.1). Although *Lactobacillus plantarum* (Rosellac C) and C 4 were inhibitory to some indicator strains, *Pediococcus cerevisiae* (Hansen) and isolate #26 produced inhibitor(s) active against a wide spectrum of microorganisms.

4.3 Spectrum of Activity

The inhibitory spectra of isolate #26 and *P. cerevisiae* (Hansen) are illustrated in Table 4.1. Most gram-positive organisms, including species of *Pediococcus*, *Lactobacillus*, *Leuconostoc*, *Streptococcus*, *Clostridium*, *Bacillus* and *Listeria* are susceptible to the inhibitor(s) produced by isolate #26 and *P. cerevisiae* (Hansen); however, none of the gram-negative organisms (see Table 3.2) examined is inhibited. Neither isolate #26 nor *P. cerevisiae* (Hansen) synthesized an inhibitor which was active against itself when tested by the direct and deferred procedures, thus these strains are immune to at least nominal levels of their own inhibitor(s). Although *P. cerevisiae* (Hansen) produced a potentially useful antimicrobial system, it was not examined further due to its inability to grow at low temperatures. Some strains, including *S. faecalis*, *L. mesenteroides* and *L. viridescens* are particularly susceptible to the inhibitory substance(s) released by isolate #26; therefore, these

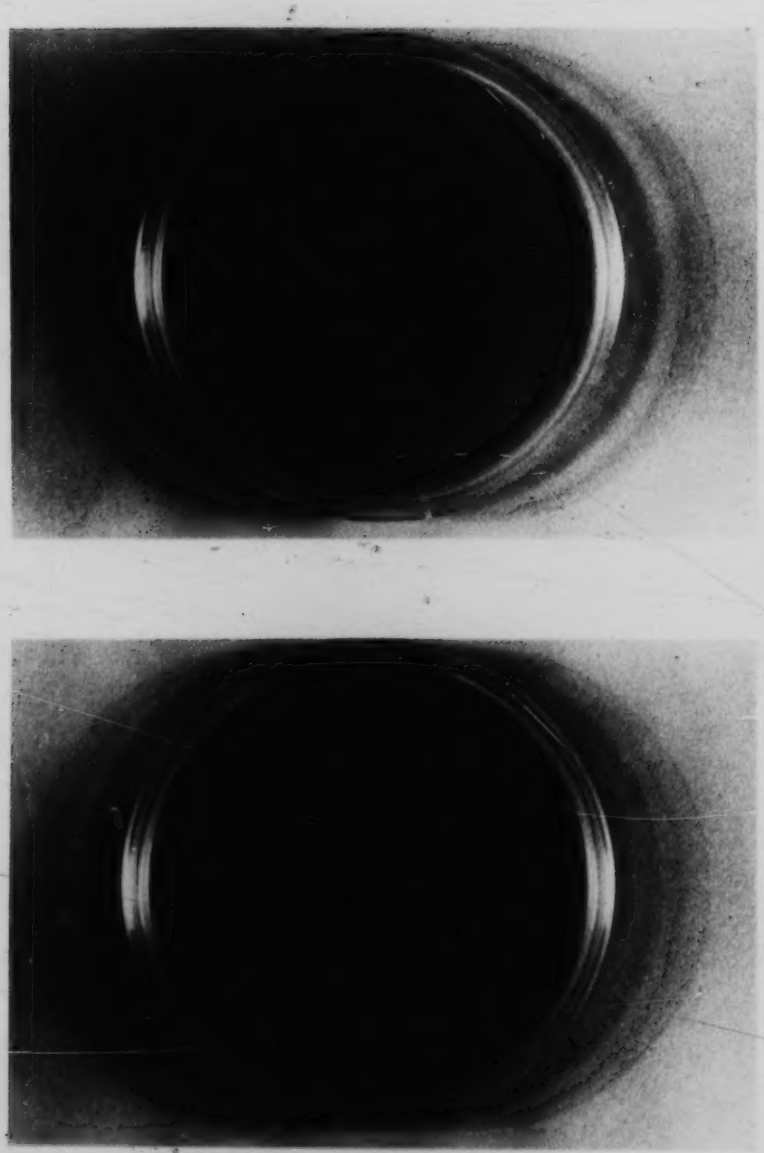


Figure 4.1. Direct (A) and deferred (B) antagonism tests showing inhibition of a lawn culture of *Streptococcus faecalis* ATCC 7080 by isolate #26.

Table 4.1. Activity spectra of isolate #26 and *Pediococcus cervisiae* (Hansen).

Indicator organism	Sensitivity to strain[1]	
	#26	PCH
Pediococcus spp.		
P. cerevisiae (Hansen)	+	-
P. cerevisiae (Lactocel)	-	-
P. pentosaceus	+	+
P. acidilactici	+	±
P. parvulus	+	+
C 4	+	-
C 35	+	+
C 43	+	+
Lactobacillus spp.		
L. viridescens	+	+
L. plantarum	+	+
L. plantarum (Rosellac C)	+	-
Lactobacillus R.S.M.	+	+
Leuconostoc spp.		
L. mesenteroides	+	+
Streptococcus spp.		
S. faecalis	+	+
S. bovis	+	-
Bacillus spp.		
B. cereus ATCC 14579	+	+
B. cereus NCDO 577	-	-
Clostridium spp.		
C. perfringens	+	+
Listeria spp.		
L. monocytogenes ATCC 15313	+	+
L. innocua	-	-
L. monocytogenes 81-861	-	-
L. monocytogenes 85-307	-	-
Staphylococcus spp.		
S. aureus	-	-
S. epidermidis	-	-

[1] Producer strains demonstrated bacterial antagonism by direct and deferred antagonism procedures.
PCH = *Pediococcus cerevisiae* (Hansen).

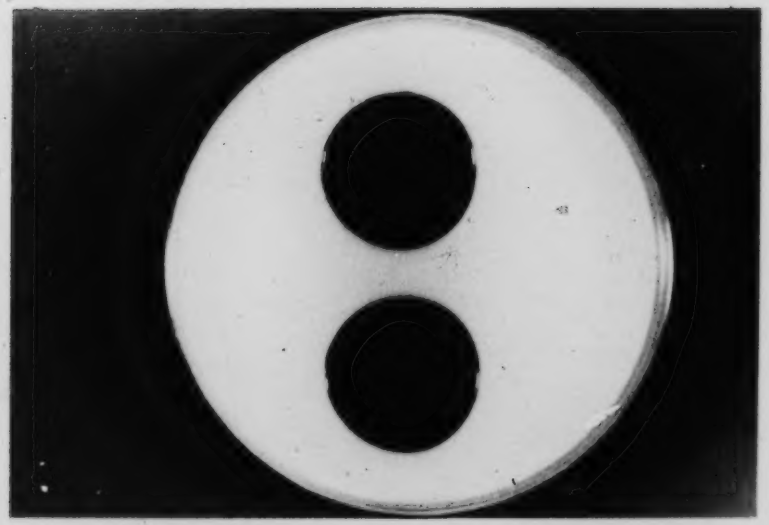
strains were selected for use as indicator strains in many of the subsequent analyses.

4.4 Distinction from other Bacterial Inhibitors

Tests were performed to exclude unrelated phenomena that may mimic bacteriocin-like antagonism on solid agar. When bacteriophage infectivity testing was performed with agar cut from the inhibitory zones, there was no plaque formation, demonstrating that the inhibitor was not a self-replicating infective entity. The inhibitor diffused through the agar, as evidenced by zones of inhibition in the indicator overlay on the reverse side of an APT culture plate. Production of the inhibitor during anaerobic incubation showed that the inhibitor was not hydrogen peroxide. This was supported by the finding that 68 units of catalase/mL incorporated into the agar did not interfere with the inhibitory effect.

Inhibition of *L. mesenteroides* and *L. viridescens* by isolate #26 inoculated into APT agar containing 68 units of catalase/mL and also calcium carbonate (2% w/v) is illustrated in Figure 4.2. The effect of acidity was neutralized by the addition of calcium carbonate to the agar, indicating that the inhibition is not due to organic acids. The exclusion of bacteriophage, hydrogen peroxide and organic acids as explanations for the inhibitory effect, gives evidence to suggest that the inhibitor produced by isolate #26 is bacteriocin-like (Tagg *et al.*, 1976). This

A



B

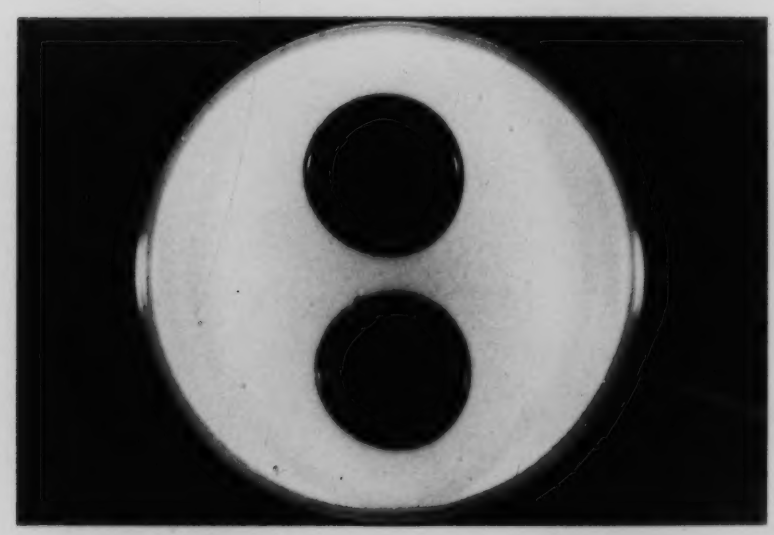


Figure 4.2. Agar well method of Dahiya and Speck (1968) demonstrating (A) inhibition of *L. viridescens* ATCC 12706 (top) and *L. mesenteroides* ATCC 23368 (bottom) on APT inoculated with isolate #26, catalase (68 U/mL) and calcium carbonate (2% w/v) as illustrated by a black ring surrounding these cultures and (B) no inhibition on the uninoculated control plate.

bacteriocin-like inhibitor is subsequently referred to as Bac #26.

4.5 Conditions Affecting Bac #26 Production on Solid Media

Plates of APT agar adjusted to pH 5.0, 5.5, 6.0, 6.5, 7.0 and 7.5 with 1 N HCl or 85% lactic acid and with 1 N NaOH were compared as substrates for the production of Bac #26 using the deferred antagonism technique. Growth of isolate #26 was poor on plates adjusted to pH 5.0 and 5.5 with 1 N HCl. There was no growth and poor growth on APT agar plates adjusted to pH 5.0 and pH 5.5 with 85% lactic acid, respectively. Growth but no zone of inhibition was observed at pH 6.0, although zones of inhibition were detected on APT agar at pH 6.5, 7.0 and 7.5. The diameters of these inhibitory zones at each pH value is given in Table 4.2.

To determine whether Bac #26 was produced during growth of isolate #26 at low temperature, isolate #26 was inoculated onto APT plates, incubated at 1, 4 and 10°C and examined for inhibitor production at 2 day intervals by the deferred antagonism technique. Production of Bac #26 was evident after 6 days at 1°C, after 4 days at 4°C and after 2 days at 10°C. The diameters of the zones of inhibition were measured at each temperature and are recorded in Table 4.3.

Table 4.2. The effect of pH on Bac #26 production on APT agar measured by the diameter of the zone of inhibition appearing in the indicator overlays.

Indicator organism	Diameter (mm) of zone of inhibition at given pH[1]			
	7.5	7.0	6.5	6.0
L. mesenteroides	15	15	14	0
S. faecalis	10	9	10	0
L. plantarum	16	15	14	0
C 43	N.D.	18	17	0
Lactobacillus R.S.M.	N.D.	14	7	0
P. pentosaceus	N.D.	10	7	0

[1] Average diameter (mm) of the zone of inhibition appearing in the indicator overlay by the deferred antagonism procedure. 0 = no inhibition. N.D. = not determined.

Table 4.3. The effect of incubation temperature on Bac #26 production on APT agar measured by the diameter of the zone of inhibition appearing in the indicator overlays.

Incubation		Indicator[1]	
Temperature	Time (days)	*L. mesenteroides*	*L. viridescens*
10°C	2	8	10
	4	19	20
	6	22	25
4°C	4	5	4
	6	12	15
	8	18	18
1°C	6	13	14
	8	18	20
25°C	1	14	11

[1] Average diameter (mm) of the zone of inhibition appearing in the indicator overlay by the deferred antagonism technique.

4.6 Enzyme Inactivation of Bac #26

When Bac #26 was tested for inactivation by protease, no zones of inhibition were observed in the indicator lawns.

4.7 Plasmid Involvement

Since most of the genetic determinants for bacteriocin production are plasmid-borne, isolate #26 was analyzed for plasmid DNA. However, attempts to demonstrate the presence of plasmid DNA were unsuccessful.

4.8 Morphological Characteristics

Cells of isolate #26 are rod-shaped, occurring singly or in pairs, and frequently joined at an angle, forming a V-shape. On APT agar, isolate #26 grows as small creamy-white colonies. The colonies are convex after anaerobic incubation at 25°C for 48 h, but after extended aerobical incubation (24 h) autolysis occurred, that is the colonies developed a central plateau with an elevated rim.

4.9 Properties of Isolate #26

To determine the identity of isolate #26, a number of metabolic, biochemical and physiological tests was performed. Isolate #26 was identified as a heterofermentative *Lactobacillus* species based on the following characterstics: Gram-positive rod; non-spore forming; fermentative growth on glucose; catalase negative; benzidine negative; oxidase negative; nitrate reduction negative;

arginine dihydrolase positive and production of CO_2 from glucose in GP agar (see Table 3.4). Gas production was evident after 5 d growth of isolate #26 in GP agar by displacement of the vaspar plug. However, gas did not accumulate in durham vials when isolate #26 was grown in GP broth.

Further properties of isolate #26 are listed in Tables 4.4, 4.5 and 4.6. In preliminary tests it became apparent that isolate #26 differed from other heterofermentative lactobacilli, in that it grows poorly on $MRS_{(5.5)}$ agar and does not grow on $acetate_{(5.6)}$ agar, media which are commonly employed for the selective isolation of this genus (Sharpe and Fryer, 1965). It also differs by producing almost exclusively (98%) of the L(+) isomer of lactic acid from glucose, reducing 0.1% (w/v) TTC and ceasing to grow at a terminal pH >4.15 in La broth. Therefore, the characteristics of isolate #26 were compared with those of *L. divergens* and *L. piscicola* (atypical species with respect to other classified betabacteria) to determine how closely related isolate #26 is to these species. In Tables 4.5 and 4.6 some of the properties of isolate #26 are listed with the properties published for representative spp. of *L. divergens* and *L. piscicola*. As shown in Table 4.6, the carbohydrate fermentation pattern of isolate #26 is the same as that given by Shaw and Harding (1985) for *L. piscicola*, which suggests that isolate #26 is a strain of *L. piscicola*.

Table 4.4. Physiological, biochemical and metabolic properties of isolate #26.

Growth on:	Acetate agar pH 5.6	-
	Lactate agar pH 5.6	-
Growth at:	pH 3.9	-
	pH 4.4	-
Growth in:	8% NaCl	-
	0.1% sodium nitrite	+
	0.05% potassium tellurite	+
	2.5% potassium thiocyanate	
	0.01% TTC	+
	0.1% TTC	+
Reduction of:	0.05% potassium tellurite	+
	0.01% TTC	+
	0.1% TTC	+
	nitrate	-
	NO_3 → amine, no gas	-
Survival at:	60°C/15 min	-
End point pH in La broth:		4.83
Lactic acid isomer:	L(+)	98%
	D(-)	2%
Hydrolysis of:	Esculin	+
Oxidate/fermentative with glucose:		F
Methyl red test:		+
Production of:	Pseudocatalase	+
	β-Glucosidase	+
	N-Acetyl-glucosaminidase	-
	β-Galactosidase	-
	Phosphatase	-
	Leucine aminopeptidase	-
	Serine aminopeptidase	-
	Pyroglutamic acid	-
	Acylamidase	-
	Aminopeptidase	-

Table 4.5. Biochemical properties of isolate #26 and those published for *L. divergens* and *L. piscicola* spp.

Property	Isolate #26	*L. divergens*[1] DSM 20623[T]	*L. piscicola*[1]
Gas production[2] from glucose	-	+	wk
Lactate isomer	L	L	L
NH_3 from arginine	+	+	+
Growth on acetate agar, pH 5.6	-	-	-
Reduction of 0.01% (w/v) TTC	+	+	+

[1] Data from Shaw and Harding (1985)

[2] Gas production from glucose determined in GP broth
T = Type strain

Table 4.6. The carbohydrate fermentation patterns of isolate #26, *Lactobacillus divergens* and *Lactobacillus piscicola*.

Fermentation of	Isolate #26	*L. divergens*[1]	*L. piscicola*[2]
Amygdalin	+	+	+
Arabinose	-	-	-
Cellobiose	+	+	+
Dextrose	+	+	+
Galactose	-	d	d
K gluconate	-	+	+
Glycerol	+	(+)	+
Lactose	-	-	d
Maltose	+	+	+
Mannitol	+	-	+
Melibiose	-	-	d
Melezitose	-	-	d
Raffinose	-	-	-
Rhamnose	-	-	-
Ribose	+	+	+
Salicin	+	+	+
Sorbitol	±	-	-
Sucrose	+	+	+
Trehalose	+	+	+
D-xylose	-	-	-
α-methyl-D-glucoside	N.D.	-	(+)

[1] Data from Kandler and Weiss 1986, Bergey's Manual of Systematic Bacteriology

[2] Data from Shaw and Harding (1985)
Symbols: d, differential; -, negative; +, positive; (+), weak positive reaction

Isolate #26 grew in BM broth at 1°C within 8 d, at 4°C within 4 d and at 15°C in 24 h, but not at 45°C. The temperature range for growth of isolate #26 was estimated using a temperature gradient incubator. Growth curves in Figure 4.3 and 4.4 show growth of isolate #26 in APT broth over temperatures ranging from approximately 1°C to 38°C. Growth of isolate #26 was poor at temperatures near 36°C.

4.10 Associative Growth of Isolate #26 and *S. faecalis* in Ground Beef

The subsequent experiments were designed to determine whether Bac #26 could be synthesized in the meat environment and, if synthesized, whether it would inhibit *S. faecalis* that had been added to the meat.

In the first trial, two sets of irradiated ground beef samples were inoculated with *S. faecalis* at 10^5 CFU/g. One set was also inoculated with isolate #26 at 10^5 CFU/g, and a third set of irradiated samples served as an uninoculated control. The samples were stored in anaerobic jars flushed with 95% H_2 and 5% CO_2 at 25°C for up to 9 days. In samples inoculated with only *S. faecalis*, growth of *S. faecalis* to 10^8 CFU/g occurred within one day. When grown in association with isolate #26 *S. faecalis* was not inhibited. Both isolate #26 and *S. faecalis* grew to approximately 10^8 CFU/g within 1 d at 25°C, after which no further change in cell density occurred (Figure 4.5).

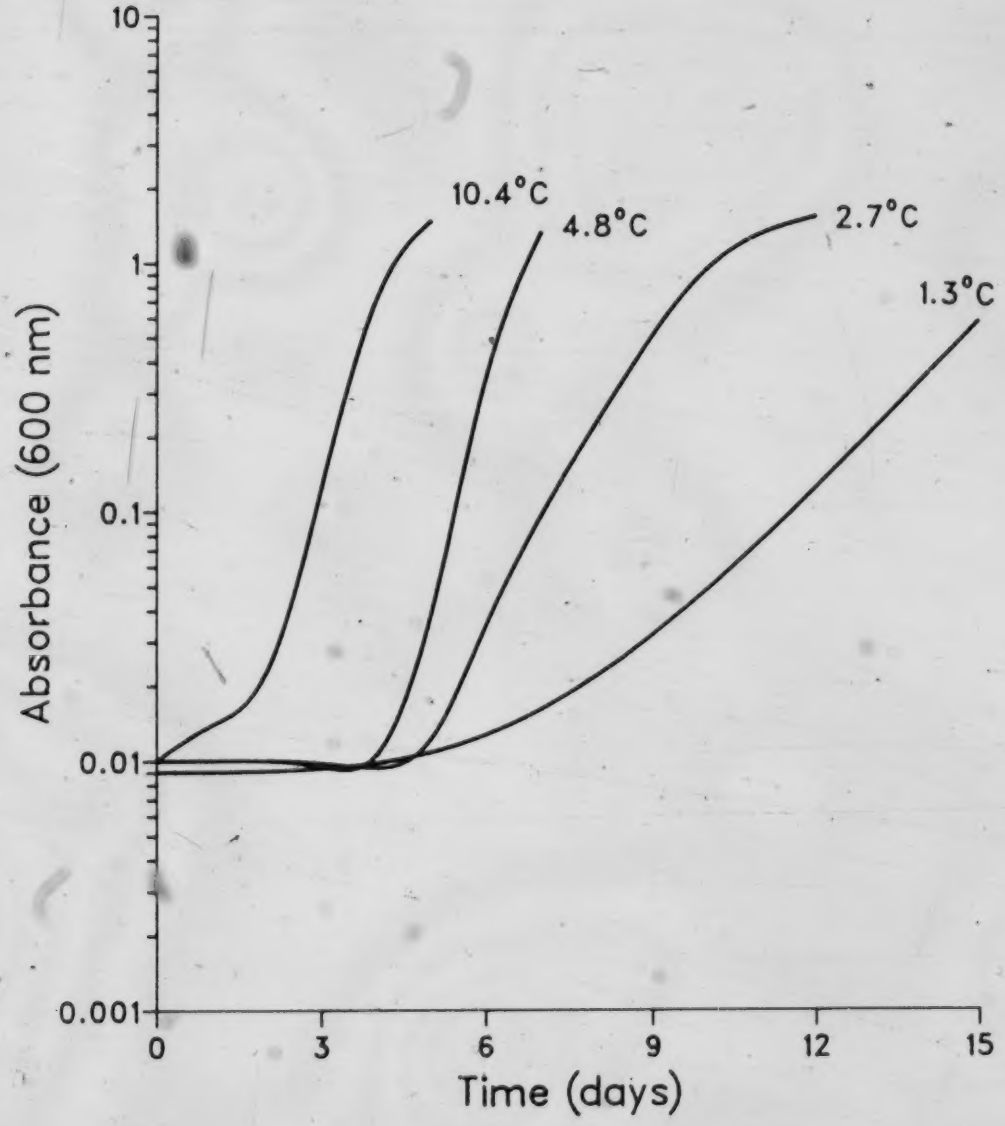


Figure 4.3. Growth of isolate #26 in APT broth measured by absorbance (600 nm) at temperatures ranging from 1.3 to 10.4°C.

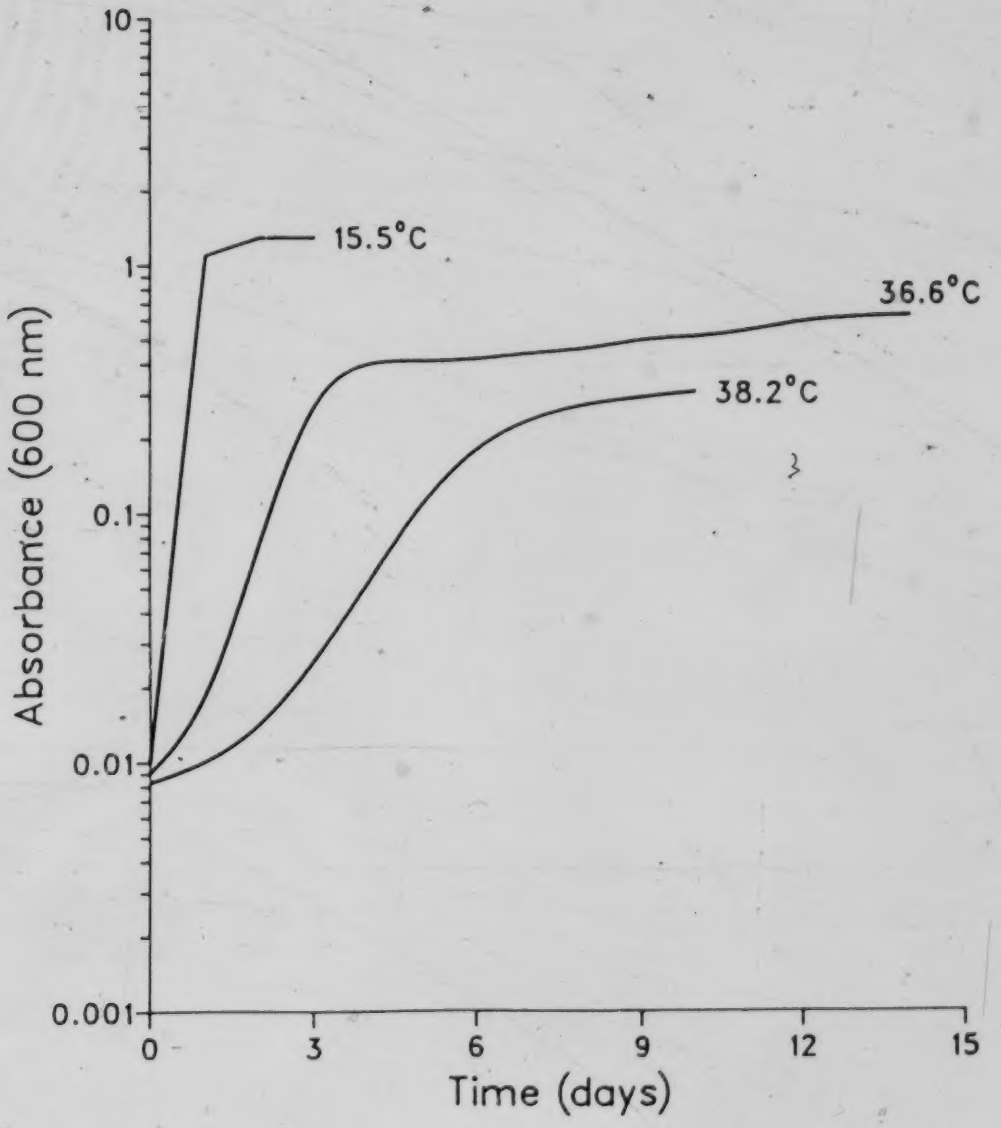


Figure 4.4. Growth of isolate #26 in APT broth measured by absorbance (600 nm) at temperatures ranging from 15.5 to 38.2°C.

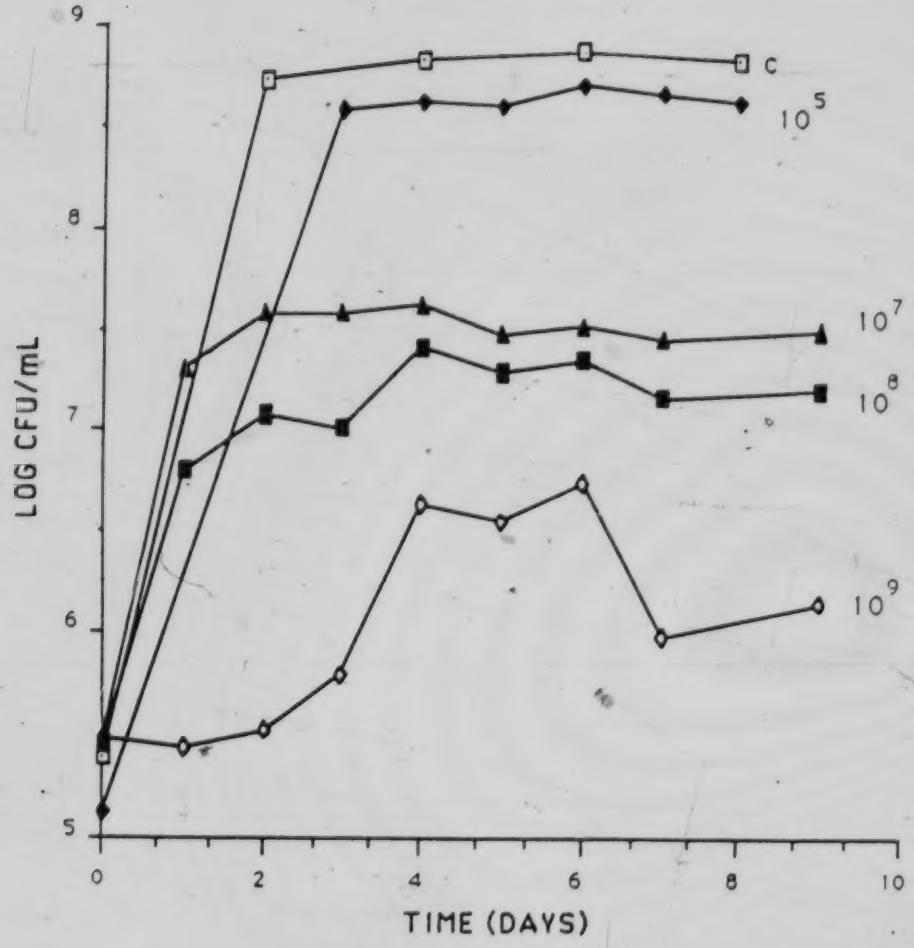


Figure 4.5. Growth of *Streptococcus faecalis* 7080 in pure culture (⊡) and in association with isolate #26 added at 10^5 (◆), 10^7 (▲), 10^8 (■) and 10^9 (◇) CFU/g in irradiated reinoculated lean ground beef stored at 25°C.

Based on the above results, a second experiment was performed to determine if growth of *S. faecalis* would be inhibited by a more concentrated inoculum of isolate #26. This experiment was identical to experiment #1, except that isolate #26 was inoculated at 10^7, 10^8 or 10^9 CFU/g. In irradiated beef samples with 10^7 or 10^8 CFU of isolate #26/g, the numbers of *S. faecalis* plateaued approximately one log cycle less than was observed in the control samples (Figure 4.5). When *S. faecalis* was inoculated with isolate #26 at 10^9 CFU/g it was almost completely inhibited. There was however a sudden increase in numbers of *S. faecalis* at day 4, followed by a sudden decrease at day 7, as illustrated in Figure 4.5.

Although *S. faecalis* does not grow at 4°C, a subsequent trial was carried out at this temperature to determine whether non-growing cells were sensitive to the bacterial inhibitor produced by isolate #26. Three sets of irradiated ground beef were inoculated with *S. faecalis* at 10^5 CFU/g. Two sets were also inoculated with 10^5 and 10^9 CFU/g of isolate #26. A set of uninoculated, irradiated samples was also incubated at 4°C. In the *S. faecalis* control samples and in the samples inoculated with *S. faecalis* and isolate #26 at 10^5 or 10^9 CFU/g, there was no decline in the numbers of *S. faecalis* over the 7 d study. For example, the log CFU/g on KF agar was 5.2, 5.3 and 5.2 at day 0, and 5.3, 5.2 and 5.3 at day 7 in samples inoculated with only *S. faecalis*, with 10^5 and with 10^9 CFU of isolate #26/g,

respectively.

4.11 Associative Growth of Isolate #26 and *S. faecalis* in Nutrient Broth

Nutrient broth was used as a substrate for associative growth of isolate #26 and *S. faecalis* to determine whether the inhibitory substance could be produced in a medium without a carbohydrate source. Three sets of nutrient broth were inoculated with *S. faecalis* at 10^5 CFU/mL, and two of these were also inoculated with isolate #26 at 10^5 or 10^9 CFU/mL. Growth of *S. faecalis* was almost completely inhibited at 25°C in association with isolate #26 at 10^5 CFU/mL (Figure 4.6). When grown in association with a isolate #26 at 10^9 CFU/mL, little if any inhibition was observed by the end of the 7 day study period, also illustrated in Figure 4.5.

4.12 pH of the Beef Samples and of the Nutrient Broth

As shown in Table 4.7, initial pH values of the ground beef samples varied from 5.6 to 5.8. The pH decreased to 5.1 in all samples inoculated with a mixed culture (*S. faecalis* plus isolate #26 at 10^5, 10^7, 10^8 or 10^9 CFU/g) by day 1, after which there was no further decline in pH.
In samples inoculated only with *S. faecalis*, the pH levels decreased to 5.2. In the uninoculated control samples, the pH levels decreased to 5.2 by day 2 of the study and to pH 5.0 by day 3, after which there was no further decline.

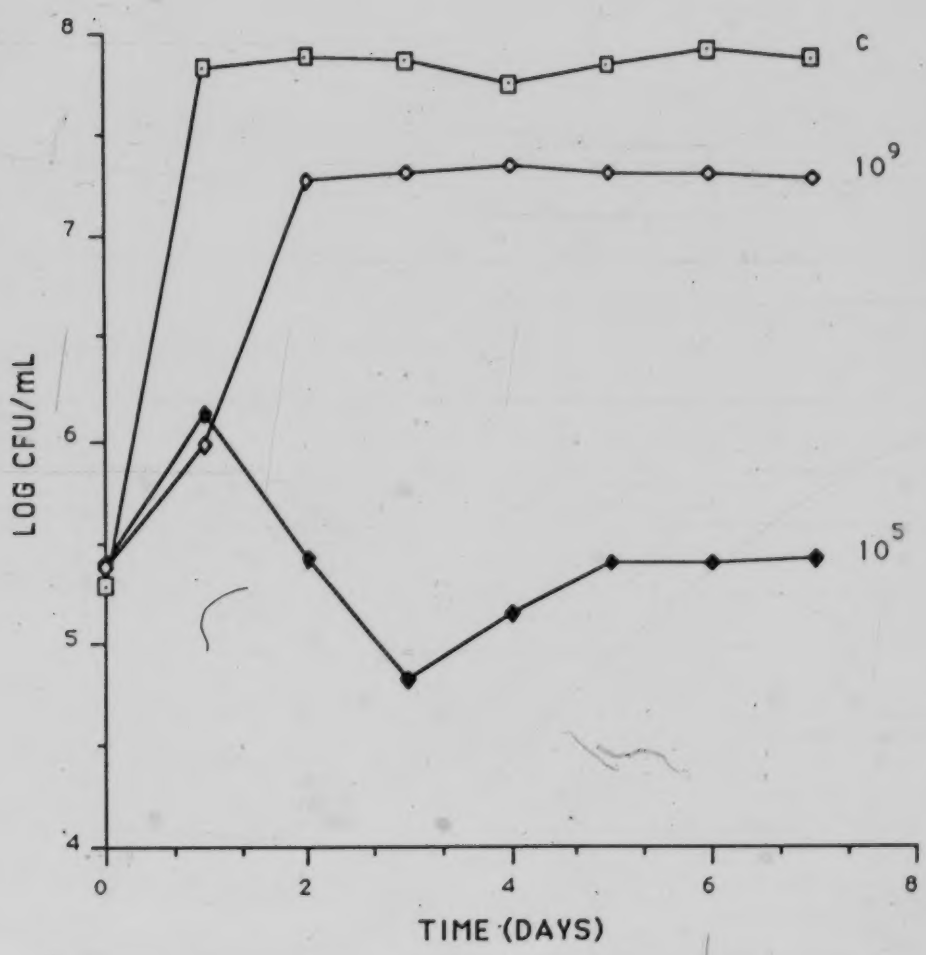


Figure 4.6. Growth of *Streptococcus faecalis* 7080 in pure culture (⊡) and in association with isolate #26 added at 10^5 (◇) and 10^9 (◆) CFU/g in nutrient broth stored at 25°C.

Table 4.7. The pH of ground beef inoculated with *S. faecalis* 7080 and isolate #26 during anaerobic storage at 25°C for 9 days.

Sampling Day	Inocula[1]					
	SF	26(5)	26(7)	26(8)	26(9)	U
0	5.8	5.7	5.7	5.7	5.6	5.7
1	5.2	5.1	5.2	5.1	5.2	5.7
2	5.2	5.2	5.1	5.1	5.1	5.2
4	5.2	5.1	5.0	5.1	5.1	5.0
6	5.2	5.1	5.0	5.0	5.0	N.D.
9	5.2	5.1	5.1	5.1	5.1	5.1

[1] Abbreviations:
SF = 10^5 CFU of *Streptococcus faecalis*/g
26(n) = 10^5 CFU of *S. faecalis* + 10^n CFU of isolate #26/g
U = uninoculated control
N.D. = not determined

Table 4.8. The pH of Nutrient Broth inoculated with *Streptococcus faecalis* 7080 and isolate #26 stored at 25°C for 7 days.

	Inocula[1]		
Day	SF	26(5)	26(9)
0	6.6	6.6	6.6
2	6.6	6.6	6.5
3	6.6	6.6	6.6
4	6.6	6.6	6.5
5	6.6	6.6	6.3
6	6.6	6.5	6.4
7	6.6	6.6	6.4

[1] Abbreviations:
SF = 10^5 CFU of *Streptococcus faecalis*/g
26(n) = 10^5 CFU of *S. faecalis* + 10^n CFU of isolate #26/g

The initial pH of nutrient broth was 6.6, as indicated in Table 4.8. A decline in pH occurred only in tubes inoculated with 10^9 CFU of isolate #26/mL. In these tubes the pH values decreased to 6.3-6.4.

5. DISCUSSION AND CONCLUSION

This study was done to determine if lactics which proliferate on vacuum-packaged meat during chilled storage could be used as biological preserving agents. During slaughter procedures and processing, particularly during grinding of fresh meat, microbial contamination occurs. The storage life of meat is highly dependent on the initial microbial load (Ingram, 1972) and it has often been demonstrated that keeping quality is first and foremost affected by hygienic practices (Childers *et al.*, 1973; Smulders and Woolthuis, 1983). Despite good hygienic measures, meat becomes contaminated during preparation. Additional means are required to retard spoilage and eliminate pathogenic microorganisms, and thus improve the quality and shelf-life of such products.

Domination of the microflora by the lactobacilli in vacuum-packaged meat is generally considered desirable. Their presence contributes to the extension of shelf-life (up to eight weeks or more) achieved by vacuum-packaging and chilled storage (Dainty *et al.*, 1979; Egan, 1983). The lactics contribute to the extension of shelf-life by successfully competing for growth space and by the production of compounds antagonistic to the growth of other microbes (Blickstad, 1983). By extrapolation of in vitro interactions (Upreti and Hinsdill, 1975; Newton and Gill, 1978; Dubois *et al.*, 1979) it seems reasonable to suggest that bacteriocins or antibiotic-type compounds play a role

in the development and maintenance of bacterial populations within vacuum-packaged meats. It follows that among the lactobacilli proliferating on vacuum-packaged meat there may be strains which are suitable for use as biological preservatives.

Isolate #26 was identified as *L. piscicola*. This is not surprising since the lactobacilli, often atypical species, predominate at chill temperatures on vacuum-packaged beef (Hitchener *et al.*, 1982; Holzapfel and Gerber, 1983; Shaw and Harding, 1984, 1985). By the production of L(+) lactic acid from glucose, isolate #26, like *L. divergens* and *L. piscicola*, differs from formally recognized species of beta-bacteria which produce DL lactic acid (Kandler and Weiss, 1986). If necessary the identity of isolate #26 could be confirmed by shoving the absence of lactobacillic acid in the cellular fatty acids (Shaw and Harding, 1985), by determining its mol% G+C and by DNA hybridization with *L. piscicola* spp.

Division of the lactobacilli into homofermentative and heterofermentative species is based on detection of CO_2 produced by the heterofermenters (Sharpe *et al.*, 1966). Isolate #26 is considered to be heterofermentative, however inconsistencies in gas production were apparent between agar and broth forms of the test medium. No visible gas was formed in Durham tubes for periods up to 10 days when isolate #26 was grown in broth. Shaw and Harding (1985), observed no gas production or very small quantities of gas

produced by *L. piscicola* isolates grown in broth.

The methods used for classifing the meat lactics are based on properties of organisms from quite different environments (Kitchell and Shaw, 1975). Certain procedures may not be satisfactory for classifying the lactobacilli isolated from vacuum packaged beef, unless modifications are made. For example, production of CO_2 is normally determined using the method of Gibson and Abdel-Malek (1945). Using this method (i.e with a high glucose, milk based medium) Shaw and Harding (1984) concluded that strains from cluster I (now known to contain isolates belonging to *L. piscicola* and *L. divergens*) were homofermentative. The heterofermentative capability of these isolates was confirmed in a subsequent study (Shaw and Harding, 1985) by analysis of their metabolic end products, which included acetate and ethanol. However, the molar ratios of ethanol and acetate and CO_2 to lactate were extremely low in both *L. divergens* and *L. piscicola* compared with typical heterofermenters. Shaw and Harding (1985) concurred with the suggestion first made by Holzapfel and Gerber (1983) that glucose fermentation by these organisms differs from the traditional pathway of the heterofermentative lactobacilli.

Various methods of direct and deferred antagonism were investigated. The direct procedure developed for this study differs from other direct procedures (Tagg *et al.*, 1973; Upreti and Hinsdill, 1973; Barefoot and Klaenhammer, 1983) in that the indicator lawn was not seeded before inoculation

of the test strain. Since a large number of test strains, approximately 100, was screened for antagonism, addition of the indicator organisms seeded in an agar overlay, following inoculation of the test cultures by a replicate plater, was felt to be more time efficient and to give more clearly defined inhibitory zones than the other procedures investigated.

In both the deferred and direct antagonism tests, isolate #26 inhibited a wide range of gram-positive organisms on APT agar. Antagonism of the growth of one bacterium by another may result from a number of different mechanisms, including: (i) the release of bacterial inhibitors such as bacteriocins, bacteriophage, hydrogen peroxide, or organic acids; (ii) the establishment of a restrictive physiological environment by alteration of factors such as pH or redox potential; and (iii) depletion of essential nutrients or growth factors (Tagg *et al.*, 1973).

Bacteriophage did not seem to be responsible for the observed inhibition since the inhibitor readily diffused through the agar (Barefoot and Klaenhammer, 1983) and the inhibitory zones were not infective. Inhibition could be demonstrated under anaerobic conditions and in the presence of catalase; therefore, hydrogen peroxide was also excluded from consideration. Under conditions which neutralized the effects of acidity, isolate #26 retained its inhibitory activity, indicating that the observed inhibition was not

caused by adverse pH conditions or by the inhibitory effects of the acid molecule itself. Inhibition of indicator cultures due to the depletion of essential nutrients or to a reduction of redox potential is unlikely. In support of this statement, the growth of *S. faecalis*, for example, was not inhibited on APT plates on which isolate #26 had previously grown and to which protease had been added. By excluding phenomena which can mimic bacteriocin activity on solid media, the inhibitory activity of isolate #26 on APT agar can be attributed to the production of a bacteriocin-like compound (Bac #26).

Inhibitory activity was eliminated on culture plates treated with protease, indicating that Bac #26 is proteinaceous in nature. However, based on the Tagg *et al.*, (1976) definition, that bacteriocins are proteinaceous in nature and have a bactericidal mode of action, Bac #26 was not confirmed to be a bacteriocin. An active extract containing Bac #26 could not be prepared from the culture supernate or from APT cultures plates of isolate #26, therefore it was not possible to determine its mode of action.

Failure to isolate active bacteriocin from the culture supernate is not uncommon. For a number of gram-positive organisms, production of bacteriocin-like substances has been demonstrated only on solid media (Tagg *et al.*, 1973; Tagg *et al.*, 1976). Tagg *et al.* (1973) suggested that failure to recover active bacteriocin from the culture

supernate may be due to its inactivation by proteases produced later in the growth of the culture. On solid agar, the bacteriocin may diffuse ahead of the active protease and so be protected from inactivation. It is not known whether strains of *L. piscicola* produce proteases which would inactivate a bacteriocin in broth culture. Further attempts were made to extract active bacteriocin from APT culture plates using the method and modifications of the method proposed by Barefoot and Klaenhammer (1983). Although extracts with active Lactacin B were prepared using this procedure (Barefoot and Klaenhammer, 1983), the inhibitory activity of Bac #26 was not retained in these culture extracts. It is possibile that Bac #26 was inactivated by the purification procedures or that it was not effectively extracted from the agar.

The factors controlling production of bacteriocins are poorly understood, which is complicated by the fact that not all cells in a particular culture produce bacteriocins at the same time or under the same circumstances (Tagg *et al.*, 1976). Variation in composition of the media, as well as conditions such as time, aeration, temperature, and pH can have profound effects on the yield of active bacteriocin (Tagg *et al.*, 1976). Although initial attempts to isolate Lactacin B from the culture supernate were unsuccessful, Barefoot and Klaenhammer (1984) found that by growing the cells under optimal conditions significant activity was detected. They found maximum titers of activity at pH 6.0

after growth of the culture for 10 h, but that activity decreased by 75% within 4 h. Thus, it is possible that Bac #26 could also be partialy purified from the culture supernate by systematic research to determine the optimal conditions for its production in broth.

The initial pH of the APT agar was found to be an important factor in determining whether Bac #26 is produced. The diameters of the zones of inhibition produced by isolate #26 on APT at pH 6.5, 7.0 and 7.5 were very similar, suggesting Bac #26 production occurs equally well at these pH levels. Although isolate #26 grows on APT at an initial pH of 6.0 and 5.5, there was no inhibitory activity, suggesting that Bac #26 is not produced or that it is inactivated at pH values below 6.0.

Lactacin B production by *Lactobacillus acidophilus* was also reported to be dependent on the initial pH of the agar (Barefoot and Klaenhammer, 1984). Activity was not detected if the initial pH was below 5.9. However, maximum production of Helveticin J (Joerger and Klaenhammer, 1986) was reported at pH 5.5. It should also be noted that the optimal pH for production of bacteriocin is not necessarily the pH at which the bacteriocin is stable. As mentioned previously, maximum production of Lactacin B occurred at pH 6.0, but at this pH Lactacin B was unstable. In this case, activity was stabilized by adjusting the pH to 5.0. (Barefoot and Klaenhammer, 1984).

Generally, bacteriocin production is greatest at a temperature optimal for growth of the producer strain (Tagg *et al.*, 1976). Growth of isolate #26 at low temperatures (1°C, 4°C and 10°C) did not affect its ability to produce Bac #26, providing that it was incubated at these temperatures long enough for sufficient growth of isolate #26 to occur.

Most of the genetic determinants for bacteriocin production are plasmid-borne (Tagg *et al.*, 1976). However, attempts to isolate plasmid DNA from isolate #26 by various protocols, including both large and small scale extraction, were unsuccessful (personal communication, Stiles and Cerrone, 1987). Isolate #26 does not appear to contain plasmid DNA, suggesting that the genetic determinant for Bac #26 is chromosomally located. Demonstrating stability of bacteriocin production under conditions that induce plasmid curing would support this suggestion. As indicated by Joerger and Klaenhammer (1986), until genes for the production of bacteriocin are actually localized in the chromosome, there remains the remote possibility that a stable, undetected plasmid is responsible for bacteriocin production. When *L. helviticus* was grown in the presence of sodium dodecyl sulfate, rifampin, ethidium bromide, acridine orange, neutral acriflavin and at higher temperatures it maintained the ability to produce Lactocin 27 (Upreti and Hinsdill, 1975). This suggests that the genetic determinants for Lactocin 27 production are not plasmid-borne. Other

attempts to demonstrate plasmid-mediated bacteriocin production in the lactobacilli have also been unsuccessful (Barefoot and Klaenhammer, 1983; Joerger and Klaenhammer, 1986). Chromosomal determinants for bacteriocin production may therefore be common among the lactobacilli. A strain with a chromosomal determinant for bacteriocin production, rather than a plasmid-borne determinant, may be more suitable for use as a biological preservative. Strains that carry plasmid DNA have longer generation times, which could affect their ability to dominate the microflora.

Isolate #26 was the only strain among isolates from vacuum-packaged meat which produced a bacteriocin-like inhibitor. Although this was the only isolate, suggesting low numbers of bacteriocin producers, the incidence of such strains in vacuum-packaged meat cannot be calculated due to the random nature in which the isolates were selected in this study.

Bacteriocin production has been reported for other lactobacilli, including strains of *L. acidophilus* (Barefoot and Klaenhammer, 1983), *L. fermenti* (DeKlerk and Smit, 1967), and *L. helveticus* (Upreti and Hinsdill, 1975; Joerger and Klaenhammer, 1986), but there have been no previous reports on production of bacteriocin-like compounds by lactics isolated from vacuum-packaged meat, presumably, because bacteriocin production has never specifically been examined among these organisms.

Bac #26 inhibited a broad spectrum of gram-positive organisms, but it was not active against any of the gram-negative organisms tested. Bac #26 differs from all previously reported *Lactobacillus* bacteriocins with respect to its broad activity spectrum. The activity spectrum for other *Lactobacillus* bacteriocins, Lactacin B (Barefoot and Klaenhammer, 1983), Lactocin 27 (Upreti and Hinsdill, 1975), and the *L. fermentum* bacteriocin (DeKlerk and Smit, 1967), is restricted to species within the genus *Lactobacillus* and with Helveticin J (Joerger and Klaenhammer, 1986) restricted further, to only closely related species. The spectrum of activity for Bac #26 is not unique when compared with the spectra cited by Tagg *et al.* (1976) for other gram-positive organisms. In fact the range of organisms inhibited by Bac #26 is almost identical to Pediocin A (Daeschel and Klaenhammer, 1985). Isolate #26 demonstrated host cell immunity to Bac #26. As indicated by Joerger and Klaenhammer (1986), the immunity of the producer cell to its own bacteriocin, although poorly understood, is believed to result from synthesis of a specific immune substance.

Irradiated ground beef stored anaerobically at 25°C was evaluated as a substrate for bacteriocin production by isolate #26. This study confirmed the reports of others (Reddy *et al.*, 1970; Gilliland and Speck, 1975; Abdel-Bar and Harris, 1984; Gill, 1986) that a high inoculum is required to achieve inhibitory effects. The inhibitory effects of the lactics inoculated into fresh ground beef

have been attributed to acid production (Reddy *et al.*, 1970), to a combination of H_2O_2 production and acid production (Abdel-Bar and Harris, 1984), and to H_2O_2 (Gilliland and Speck, 1975). Although growth of *S. faecalis* was inhibited, Bac #26 mediated inhibition is unlikely since the initial pH of fresh ground beef is below pH 6.0, and it is commonly reported to be 5.8. That Bac #26 was not detected on APT agar at pH <6.0, suggests that it would not be produced in fresh ground meat. Inhibition also cannot be attributed to H_2O_2 production as incubation of the meat anaerobically should preclude this possibility.

The pH of the beef declined rapidly from approximately 5.8 to approximately 5.0 in all samples inoculated with 10^7, 10^8 and 10^9 CFU of isolate #26/g, suggesting that organic acid may contribute or be solely responsible for the observed inhibition. Inhibition by organic acids may depend on three factors:

(i) the effect solely of pH,

(ii) the extent of dissociation of the acid, and

(iii) a specific effect related to the acid molecule (Smulders *et al.*, 1986).

There is disagreement among researchers on the relative importance of each of these factors. Gill and Newton (1975) suggested that lactic acid exerts an antimicrobial effect only by reducing pH. Other investigators (Grau, 1981; Woolthuis and Smulders, 1985) believe that the undissociated form of the acid molecule is also of significance. Grau

(1981) showed that both the lactate content and the pH of beef were important factors in controlling the growth of some gram-negative bacteria and *B. thermospacta*. Under the same conditions of pH and acid dissociation there are differences in the antimicrobial action of various organic acids (Smulders *et al.*, 1986). For example, growth of *Salmonella gallinarum* was inhibited by undissociated acetic acid, but undissociated lactic acid was only slightly inhibitory (Sorrells and Speck, 1970).

If inhibition is solely the result of acid production, it is unclear why inocula of 10^7 and 10^8 CFU of isolate #26/g do not inhibit *S. faecalis* to the same extent as an inoculum of 10^9 CFU/g. An inoculum of 10^9 CFU of isolate #26/g may reduce the oxidation-reduction potential to a level which contributes to the increased inhibition of *S. faecalis*, or may cause increased inhibition by limiting the "space" available for growth of *S. faecalis*, that is, by creating a physical barrier.

Inoculation of isolate #26 and *S. faecalis* into nutrient broth illustrates further how the effect of a particular culture may vary under different environmental conditions. Inhibition of the growth of *S. faecalis* was observed with an inoculum of 10^5 CFU/g but not with higher inocula. Although the pH dropped to 6.3, *S. faecalis* was able to initiate growth at this pH; therefore, inhibition caused by organic acids is unlikely. Use of a mineral overlay at the surface of the nutrient broth reduces the

likelihood of H_2O_2 as the inhibitory mechanism. Inhibition in this case may be due to production of a bacteriocin-like inhibitor, however cell-free supernatants of the broth culture were not inhibitory.

5.1 Conclusions

From this study it was concluded that among the lactics proliferating on vacuum-packaged beef, there are strains of lactobacilli which produce antimicrobial compounds that are bacteriocin-like in nature. This study suggests that isolate #26 could not be used as a biological preservative in fresh ground beef by Bac #26 mediated antagonism.

Further studies are required to determine the frequency of organisms which produce bacteriocin or bacteriocin-like compounds on vacuum-packaged beef, to determine whether there are other strains of *L. piscicola*, which produce bacteriocins, and to determine whether bacteriocins produced by other lactics isolated from vacuum-packaged beef differ from Bac #26, particularly with respect to pH sensitivity. To foster an understanding of the potential and possible limitations of bacteriocin-mediated antagonism in meats, further study on the nature of Bac #26, its mechanism of action and the conditions or factors which affect its activity should be determined.

Knowledge of the taxonomy, physiology and metabolism of the lactics associated with vacuum-packaged meat is limited. Further taxonomic study on the vacuum-packaged meat

microflora is required. Knowledge of the taxonomy is essential before a total survey of the biotechnical potential of this group of organisms can be made. Further research is also required to elucidate the nature of the biochemical pathway used by the *L. divergens* and *L. piscicola* for carbohydrate fermentation, as it appears to differ from the 6-phospho-gluconate pathway used by other betabacteria.

With any new technique for preserving foods, questions regarding consumer acceptability of the product and safety of the product will inevitably be raised. Further studies, therefore, could be directed toward determining whether the organoleptic properties of fresh meat are affected by the addition of lactic acid bacteria and also to addressing safety concerns.

Despite the most sophisticated hygienic procedures, spoilage and pathogenic microorganisms will be introduced into/onto fresh meats during preparation. The challenge remains to develop techniques such as that proposed in this study for eliminating undesirable microbes from fresh meats. Based on the diversity of the bacteriocins produced by the lactics (Barefoot and Klaenhammer, 1983; Daeschel and Klaenhammer, 1986) further examination of the meat lactics may reveal an organism that produces a bacteriocin at the pH of fresh meat. Further study may reveal strains which are more suitable for use to become biological preservatives or strains whose deficiencies might be overcome by genetic

modification.

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